Journal of Chemical Ecology, Vol. 16, No. 1, 1990

INTRODUCTION

The fifth annual meeting of the International Society of Chemical Ecology was held on June 24–27, 1988, at the University of Georgia in Athens. Four symposia were featured, highlighting progress in diverse areas of chemical ecology. They were: Sensory Behavior of Microorganisms, Chemical Ecology of Reptiles, Arthropod Sequestration of Natural Resources, and Chemical Ecology in the Aquatic Environment. The *Journal of Chemical Ecology*, the official journal of the ISCE, is dedicating this issue to the proceedings from these symposia.

The first symposium, organized by Michael D. Manson (Texas A&M University), emphasized two important aspects of microbial physiology: microorganisms display an astonishing diversity of responses to environmental cues, and the molecular and cellular bases of these behaviors often are similar to those observed in multicellular organisms.

The first paper introduces bacterial motility and chemotaxis, emphasizing recent studies on signal transduction. Other papers address the physical constraints on motility and chemoreception in bacteria, chemical communication in gametes of the aquatic slime mold *Allomyces*, and the mechanism of aggregation in slime mold amebae. Also included are abstracts of talks about the evolution of bacterial signal transducers, chemical interactions between *Rhizobium* and legume roots, motility and chemotaxis in spirochetes, control of bioluminescence in marine bacteria, fruiting body formation in *Myxobacteria*, and ion channels in *Paramecium*, yeast, and bacteria.

The second symposium, organized by Paul J. Weldon (Texas A&M University), is concerned with both chemical production and chemical perception by reptiles. All extant reptiles produce skin or skin gland secretions for intraspecific communication, predator deterrence, or other functions. New information is presented on the components and possible functions of epidermal and skin gland secretions from lizards and crocodilians.

The studies of reptile chemical perception focus on the ability of squamate reptiles, specifically lizards and snakes, to identify chemicals from prey and predators. Several contributions are concerned with snakes' responses to prey chemicals, where progress is described in identifying the source and nature of chemical releasers of feeding behavior and in relating chemical perception to feeding ecology. A Letter to the Editor discusses terms to denote chemical perception by various vertebrate chemosensory organs.

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The third symposium, organized by Murray S. Blum (University of Georgia), is concerned with the abilities of species of Lepidoptera, Coleoptera, Hemiptera, Diptera, Hymenoptera, and Orthoptera to selectively sequester plant compounds and the metabolic transformations accompanying the process of compound storage. Both specific and nonspecific sequestration were examined, especially for species that exhibit both types of biomagnification.

The varied roles of sequestrable compounds were analyzed in terms of the adaptive significance for herbivores specializing on plants fortified with "toxic" natural products. A number of contributions to this symposium indicate that, in addition to acting as feeding deterrents to predators, these secondary compounds and/or their metabolites possess other diverse functions. The adaptiveness of sequestration for specialist insect herbivores is manifest by the utilization of plant-derived chemicals as pheromones, growth regulators, and oviposition and feeding stimulants.

The fourth symposium, organized by Donald J. Gerhart (Duke University), features chemical defense, chemical communication, and allelopathic competition in freshwater and marine environments. These papers describe the influence of isothiocyanates on interactions between small crustacean grazers and some aquatic macrophytes, the roles played by peptides in chemical signaling and chemoreception by marine invertebrates, and long-term studies of the chemical ecology of benthic invertebrates on the Great Barrier Reef.

Each invited symposium paper has undergone peer review in accordance with the standard policy of the *Journal of Chemical Ecology*. We are grateful to the reviewers for their valuable services.

We acknowledge the cooperation of the Plenum Publishing Corporation in producing extra copies of this issue to accommodate registrants of the meeting and others interested in the symposia proceedings.

R. M. Silverstein
J. B. Simeone, Editors
Journal of Chemical Ecology

Murray S. Blum
Donald J. Gerhart
Michael D. Manson
Paul J. Weldon, Proceedings Coeditors

Journal of Chemical Ecology, Vol. 16, No. 1, 1990

ANALYSIS OF GULAR AND PARACLOACAL GLAND SECRETIONS OF THE AMERICAN ALLIGATOR (*Alligator mississippiensis*) BY THIN-LAYER CHROMATOGRAPHY

Gland, Sex, and Individual Differences in Lipid Components

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Abstract—Secretions from the gular and the paracloacal glands of adult male and female American alligators (*Alligator mississippiensis*) were extracted with chloroform, weighed, and analyzed by thin-layer chromatography. In both sexes, more lipoidal secretions were recovered from the paracloacal glands than from the gular glands. Females produce more lipoidal secretions in both sets of glands than do males. The thin-layer chromatograms of extracts from both glands exhibit bands consistent with aliphatic alcohols, sterols, free fatty acids, and steryl esters. Triglycerides, hydrocarbons, phosphatidylethanolamine, and lysophosphatidylcholine also are indicated in some chromatograms. Gland, sex, and individual differences are suggested in the thin-layer profiles.

Key Words—*Alligator mississippiensis*, gular glands, paracloacal glands, lipids.

INTRODUCTION

The American alligator (*Alligator mississippiensis*), as with other extant crocodilians, possesses two prominent pairs of skin glands, the gular and the paracloacal glands. The gular glands are situated on the ventral aspect of the lower jaw in folds of the skin adjacent to each mandibular ramus (Weldon and Sampson, 1988). The paracloacal glands are embedded in the walls of the cloaca,

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and they release secretions through duct openings near the vent (Weldon and Sampson, 1987).

Either or both pairs of skin glands may be everted when alligators are physically restrained immediately after capture (Weldon, personal observation), but the function(s) of the secretions, and even the conditions under which they are discharged, is uncertain. Immature alligators respond by increased gular pumping to airborne presentations of gular and paracloacal gland exudates from adult males (Johnsen and Wellington, 1982), lending support to the frequent suggestion that these materials contain pheromones. Neill (1971) dismissed accounts that secretions from *A. mississippiensis* skin glands could be detected by humans. Vliet (1989), however, states that this species' secretions linger in the air after the head-slapping display and that an oily sheen, presumably from the paracloacal glands, appears on the surface of the water.

Analyses by gas chromatography–mass spectrometry (GC-MS) of glandular secretions from *A. mississippiensis* indicated that the paracloacal glands contain cholesterol, free fatty acids, a variety of esters, and α-tocopherol (Weldon et al., 1988) and that the gular glands contain cholesterol, free fatty acids, α-tocopherol, and squalene (Weldon et al., 1987). Thin-layer chromatography (TLC) and carbon-13 nuclear magnetic resonance of the gular gland secretions indicated steryl esters, triglycerides, and other compound classes (Weldon and Sampson, 1988).

We report here the results of TLC analyses of the gular and paracloacal gland secretions from adult *A. mississippiensis*. This study documents differences in the chemicals produced in these glands, provides evidence for sex and individual variation, and details the amounts of chloroform ($CHCl_3$)-soluble materials present in these exudates.

METHODS AND MATERIALS

Gular and paracloacal gland secretions were obtained from 20 male (total lengths = 1.6–2.5 m; $\bar{X}$ = 2.0 m) and 14 female (1.4–2.2 m; $\bar{X}$ = 1.8 m) alligators from Cameron parish, Louisiana, during September 1988. All specimens had been sacrificed 3–4 hr before secretions were obtained.

Secretions were collected by manually everting and compressing the capsule of the glands. Secretions from the paracloacal glands generally flowed freely from the opening of the gland duct into a vial; caseous exudates from the gular glands were scraped onto the rim of a vial or, if present as a residue around the gland duct, collected on the end of a capillary tube, which was broken off into a vial. The exudates of each pair of glands from each individual were kept separate. Approximately 1.5 ml of $CHCl_3$ were added to each vial. They were transported on ice and stored at $-90°C$.

Secretions were extracted by adding 10 ml of $CHCl_3$ to each vial, shaking for 1 min, and inserting a pipet to draw off the solvent; this process was performed twice. The extract was filtered, and the solvent was removed by rotoevaporation. The dried residue was dissolved in $CHCl_3$ and transferred to a preweighed vial. The vial was placed under a stream of nitrogen, allowed to desiccate in vacuo for 12 hr, and weighed. One milliliter of $CHCl_3$ was added to each vial, and the vials were stored at $-90°C$.

Aliquots of the gular and paracloacal gland solutions (300 μg for both polar and nonpolar lipids) were applied to 1-cm lanes on a 0.25-mm layer of silica gel 60 G on 20 × 20-cm glass plates. Secretions from eight females were applied separately on the left side of each plate; those from eight males were applied on the right side. The same individuals, spotted in the same order, were used for the chromatograms of polar and nonpolar secretions from each set of glands; different individuals were used for the analyses of gular and paracloacal secretions. Mixed lipid standards (Nu-Chek-Prep, Elysian, Minnesota, for nonpolar lipids; Supelco, Bellefonte, Pennsylvania, for polar lipids) were developed in the center lane of each plate. Separate nonpolar plates (not shown) were developed using an aliphatic alcohol (nonanol) and a hydrocarbon (squalene) as standards.

The nonpolar lipids were resolved by successive development in hexane, then toluene, and finally hexane–diethyl ether–acetic acid (80:20:2, v/v/v). The polar lipids were resolved by development in $CHCl_3$–methanol–water (65:35:5, v/v/v). The plates were sprayed with 50% sulfuric acid and heated on a hot plate to char the lipids. At least three plates of nonpolar and polar secretions from both sets of glands were examined.

RESULTS

All chromatograms of the nonpolar gular gland secretions (Figure 1) indicate bands consistent with sterols ($R_f = 0.2$), free fatty acids ($R_f = 0.4$), and steryl esters ($R_f = 0.9$); several chromatograms display a band just below that of steryl esters. A prominent band in the region to which triglycerides migrate ($R_f = 0.7$) is evident in most of the females' secretions; in males, two distinct bands (R_fs = 0.6 and 0.8) are displayed. A band at the solvent front of all chromatograms indicates hydrocarbons.

Aliphatic alcohols migrate slightly above sterols in the solvent system used here, as confirmed by tests with nonanol. One or more bands consistent with aliphatic alcohols ($R_f = 0.2$) are observed in all gular gland chromatograms.

Chromatograms of the nonpolar paracloacal gland secretions (Figure 2) display bands corresponding to sterols, free fatty acids, triglycerides, and steryl esters; all chromatograms display a band just below that of steryl esters. Two

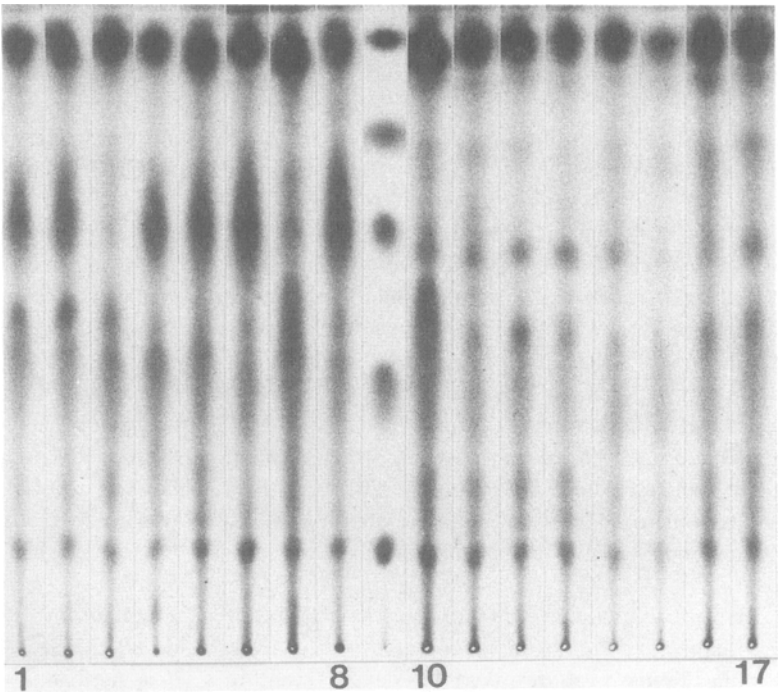

FIG. 1. Thin-layer chromatograms of nonpolar gular gland secretions from eight female (lanes 1–8) and eight male (lanes 10–17) *A. mississippiensis*. Lane 9 contains lipid standards (from bottom): sterol (cholesterol), free fatty acid (oleic acid), triglyceride (triolein), methyl ester (methyl oleate), and steryl ester (cholesteryl oleate).

prominent bands of unknown chemical classes possess R_f values of approximately 0.8 and 0.9. A faint band ($R_f = 0.1$), and others in this region, indicate the presence of compounds more polar than sterols. Another minor band ($R_f = 0.5$) also is evident. A faint band at the solvent front of several chromatograms (e.g., lane 6) is consistent with hydrocarbons.

A band in the paracloacal extracts consistent with aliphatic alcohols ($R_f = 0.2$) is evident in all chromatograms; it is more prominent in males. One male (lane 13) displays a band ($R_f = 0.3$) not apparent in the chromatograms of other individuals.

Chromatograms of the polar gular and paracloacal gland secretions (Figures 3 and 4, respectively) indicate a predominance of compounds less polar than lysophosphatidylcholine. The gular gland secretions of three females (lanes 5, 6, and 8) exhibit bands consistent with phosphatidylethanolamine ($R_f = 0.3$)

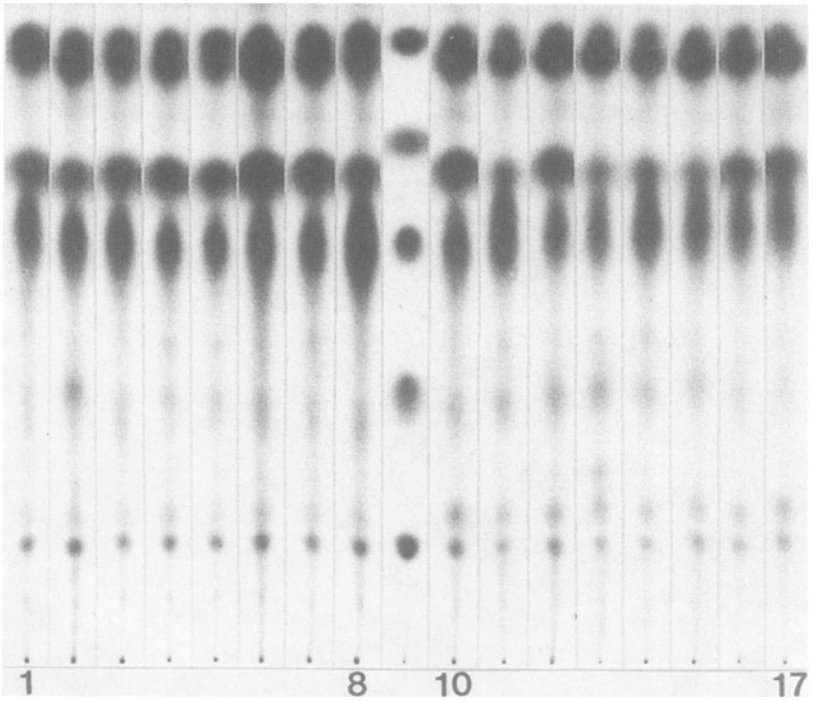

FIG. 2. Thin-layer chromatograms of nonpolar paracloacal gland secretions from eight female (lanes 1–8) and eight male (lanes 10–17) *A. mississippiensis*. Lane 9 contains the lipid standards indicated in Figure 1.

and lysophosphatidylcholine ($R_f = 0.6$); a faint band in the chromatogram of a male (lane 11) is intermediate ($R_f = 0.4$). The paracloacal gland secretions of one female (lane 7) exhibit bands consistent with phosphatidylethanolamine and lysophosphatidylcholine. One other female (lane 2) exhibits a band consistent with phosphatidylethanolamine. Other bands in this region are not apparent in the other paracloacal gland chromatograms.

The amounts of $CHCl_3$-soluble materials obtained from the gular and paracloacal gland secretions are shown (Table 1). A t test for independent samples of unequal variance (Ott, 1988) detected significantly more secretions from both the gular and the paracloacal glands of females than from those of males ($P < 0.05$ for both glands). A Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) detected significantly more paracloacal than gular gland secretions in both sexes ($P < 0.05$).

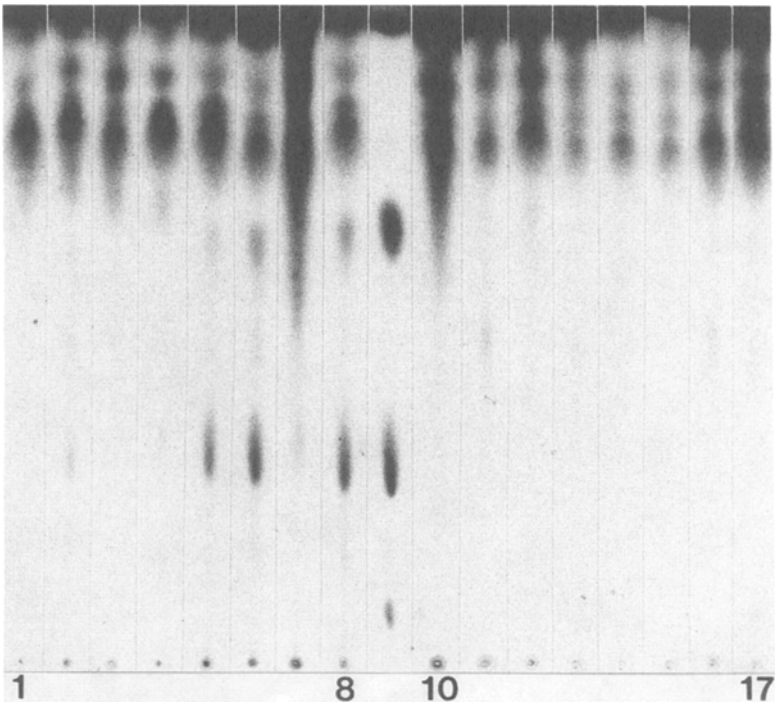

FIG. 3. Thin-layer chromatograms of polar gular gland secretions from eight females (lanes 1–8) and eight male (lanes 10–17) *A. mississippiensis*. Lane 9 contains lipid standards (from bottom): phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and sterol (cholesterol).

DISCUSSION

Differences in the gular and paracloacal gland chemicals of *A. mississippiensis* are indicated by our analysis. Nonetheless, all nonpolar TLC chromatograms of these secretions display bands consistent with sterols, free fatty acids, and steryl esters. Triglycerides are suggested in all paracloacal chromatograms and most of those of the gular gland secretions. These compound classes have been indicated in a number of TLC analyses of vertebrate epidermal or skin gland secretions (Burken et al., 1985; Stewart, 1986; Weldon and Bagnall, 1987). The structures of at least a few compounds within these classes—cholesterol and saturated and unsaturated fatty acids—have been elucidated by GC-MS analyses of the secretions of alligators (Weldon et al., 1987, 1988) and other crocodilians (Navajas Polo et al.; 1988a,b; Shafagati et al., 1989).

A TLC analysis of *A. mississippiensis* gular gland exudates by Weldon

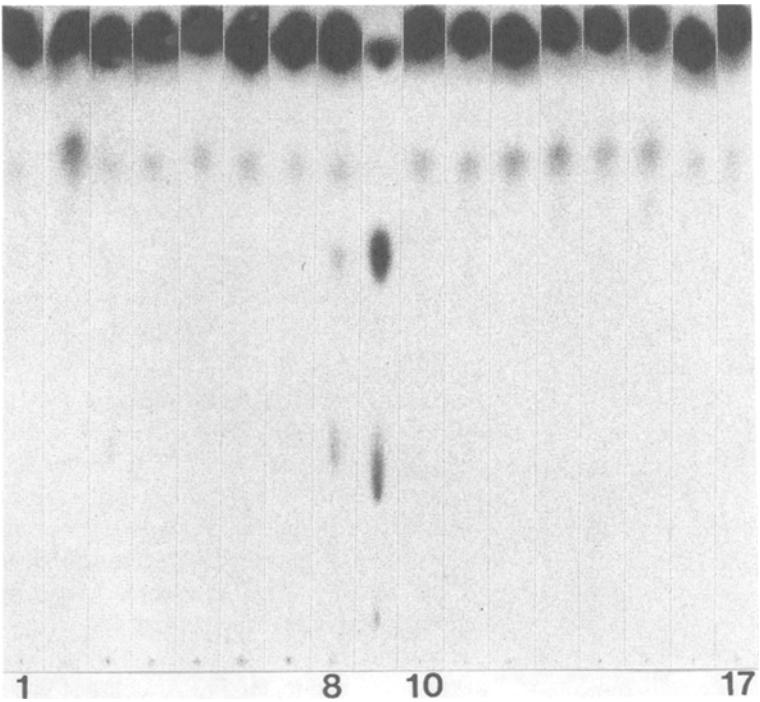

FIG. 4. Thin-layer chromatograms of polar paracloacal gland secretions from eight female (lanes 1–8) and eight male (lanes 10–17) *A. mississippiensis*. Lane 9 contains the lipid standards indicated in Figure 3.

TABLE 1. MEAN (±1 SD) WEIGHTS OF CHLOROFORM-SOLUBLE MATERIALS FROM GULAR AND PARACLOACAL GLAND SECRETIONS OF *A. mississippiensis*

	Secretion (mg)	
	Gular gland	Paracloacal gland
Male (N = 18)	$\bar{X}$ = 3.5 ± 2.6	$\bar{X}$ = 158.7 ± 158.4
Female (N = 14)	$\bar{X}$ = 27.1 ± 23.0	$\bar{X}$ = 240.5 ± 233.5

and Sampson (1988) indicated a band consistent with hydrocarbons, but this was suspected of being a contaminant. Squalene, however, has been demonstrated in a GC-MS analysis of these secretions (Weldon et al., 1987). Bands consistent with hydrocarbons occur in all gular gland and several paracloacal gland chromatograms.

A band in both the gular and paracloacal chromatograms is consistent with aliphatic alcohols. The appearance of this band in the latter exudate is noteworthy since a GC-MS analysis of paracloacal gland secretions from juvenile and adult *A. mississippiensis* failed to indicate these compounds (Weldon et al., 1988). Fester and Bertuzzi (1934) and Fester et al. (1937), on the other hand, found citronellol and other alcohols in the saponified paracloacal secretions of two caimans, *Caiman crocodilus* and *C. latirostris* (apparently pooled from both species), and Shafagati et al. (1989) found saturated and unsaturated alcohols in the secretions of dwarf (*Paleosuchus palpebrosus*) and smooth-fronted caimans (*P. trigonatus*).

In Weldon and Sampson's (1988) TLC analysis of alligator gular gland secretions, extracts were developed from three males (pooled) and one female. The chromatogram of the males displayed bands not evident in that of the female, but it was not clear whether these results reflected sex-related differences, given the small number of individuals compared. The separate development of gular gland secretions from males and females in our analysis provides evidence of sex differences in the TLC region to which triglycerides migrate. Sex differences in the paracloacal secretions are not as obvious, although the concentration of one band (possibly denoting alcohols) apparently is higher in males. Further analyses are required to determine whether sex differences exist in the polar lipids from either set of glands.

Our results indicate that the polar and nonpolar TLC profiles of gular and paracloacal glands vary among members of the same sex. The individuals we sampled ranged from approximately 5–15 years of age, judging from their lengths. Thus, all animals in our study were reproductively mature. Our results, therefore, likely reflect individual variation in the presence or concentration of gular and paracloacal gland lipids.

Measurements of the weights of $CHCl_3$-soluble substances in alligator skin glands indicate that both sexes produce more lipoidal secretions in the paracloacal glands than in the gular glands, and that females produce more lipoidal secretions in both gular and paracloacal glands than do males. Seasonal differences, however, may exist in the quantity (and quality) of glandular exudates. Bell (1827), in fact, stated that his two captive *A. mississippiensis* produced less gular gland secretions in winter than during warmer months.

Alligators in the population from which our specimens were obtained are active during September, when secretions were collected, but they mate during May and females nest during the summer. Comparisons of the amounts of secretions produced in alligator skin glands, and of the chemicals in them, at different seasons are needed.

Some authors have suggested that alligators spray secretions from the gular glands while bellowing (e.g., LeBuff, 1957). Unless, however, alligators produce substantially more gular gland materials when bellowing occurs than the

quantitites we found, the amounts of exudates in these organs probably are insufficient to be discharged in this way (even though we have weighed only the $CHCl_3$-soluble materials) (see also McIlhenny, 1935). Observations of free-ranging alligators are needed to determine the occasions upon which skin gland secretions are released.

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CHEMICAL PROPERTIES OF FEMORAL GLAND SECRETIONS IN THE DESERT IGUANA, *Dipsosaurus dorsalis*

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Abstract—This study investigates the chemistry of femoral gland secretions in the desert iguana, *Dipsosaurus dorsalis* (Lacertilia: Iguanidae), and discusses their possible functional significance. Electrophoretic and proton NMR studies indicated that the secretions are composed of approximately 80% protein and 20% lipid material. Individual differences in polyacrylamide gel banding patterns of femoral gland proteins were found. Reflectance spectroscopy revealed that the secretions strongly absorb longwave ultraviolet light, a feature that may be important in the localization of secretion deposits in the environment.

Key Words—Desert iguana, *Dipsosaurus dorsalis*, femoral glands, contact pheromones, ultraviolet light, signal localization.

INTRODUCTION

The femoral glands of lizards are follicular skin glands present in a row on the ventral surface of the hindlegs and open to the exterior through femoral pores. Femoral glands are present in many, although not all, families of lizards. Although the existence of femoral glands has been documented for over 200 years (Linnaeus, 1758), little is known of either the chemical nature of the secretion or its functional significance.

Each gland consists of several branching tubules leading to a separate pore through which a solid plug of exudate is secreted. The secretion is holocrine in nature, and, at least in some species, is composed of cells that undergo transformation and subsequently move down through the secretory duct of the glands

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where they are expelled to the outside (Cole, 1966a; Maderson, 1972). It can vary in color from white to yellowish cream, gray, or black, a feature which may be dependent on differential concentrations of melanin in the secretions of different species (Cole, 1966b). The number of pores present in the femoral series varies across species, but within a species, the glands are usually present in equal numbers in both sexes.

The microscopic anatomy of femoral glands has been described for the collored lizard, *Crotaphytus collaris* (Cole, 1966a), and for sphaerodactyline and eublepharine gekkonid lizards (Maderson, 1972). In male lizards, the glands are usually larger, more deeply embedded in the muscles of the leg, and more complex in structure, consisting of a greater number of tubules than those of females. Although the glands are evident in the embryonic stages of development, they exhibit no sexual dimorphism at this stage and do not become active until sexual maturity is reached (Cole, 1966a). The activity of femoral glands in several species reaches its peak during the breeding season (Cole, 1966b), suggesting they play some role in reproduction. The anatomical positioning of femoral glands on the underside of the hindlegs indicates that these secretions are probably passively deposited in the environment as the animal moves through its home range (Maderson, 1972; Fergusson et al., 1985).

Numerous studies have noted that treatment with androgenic hormones can induce activity of femoral glands in lizards even outside their normal breeding season (Forbes, 1941; Chiu et al., 1975; Duvall, 1979). Fergusson et al. (1985) quantitatively documented the hormonal control of femoral gland secretion in an agamid lizard, *Amphilbolurus ornatus*. They found that secretory activity in castrated males was significantly reduced but could be restored to normal levels by exogenous testosterone propionate and raised to hypernormal levels by injection of 5α-dihydrotestosterone. In females, injection of both of these androgens resulted in stimulation of secretory activity in femoral glands that had previously exhibited little or no activity. Similar results have been reported by Chiu et al. (1970) for female geckos. Seasonal activity and androgenic control of glandular activity is commonly observed in mammalian scent glands known to have communicative function (see Johnson, 1973; Ebling, 1977, for reviews).

The complex histological structure of femoral glands (Cole, 1966b), combined with their sexual dimorphism, highly seasonal activity, and control by reproductive hormones, makes it improbable that the glands are vestigial. It should be noted, however, that the glands are present in diverse taxa and occur in families spanning a wide range of ecological conditions. This makes it unlikely that the current utility of femoral gland secretions is identical in every lizard species in which they are produced. Laboratory experiments have shown that femoral gland secretions stimulate chemosensory investigation in desert iguanas, *Dipsosaurus dorsalis* (Alberts, 1989). These lizards respond to the secretions with elevated levels of tongue-touching, a chemoreceptive behavior

in which the tongue is extruded and touched to the substrate (see Simon, 1983, for review). As part of a comprehensive study of chemical signalling in *Sceloporus occidentalis,* Duvall (1986) also reported a slight increase in tongue-touches to femoral gland secretions compared to controls.

This study was undertaken to characterize the chemical nature of femoral gland secretions in *Dipsosaurus dorsalis,* an exclusively diurnal species that inhabits hot, arid desert habitats from southern Nevada, through southern California and central Arizona, into Baja California and Sinaloa in Mexico (Stebbins, 1966). Such extreme environmental conditions are expected to impose strong limitations on pheromone transmission, since volatile pheromones exhibit rapid fade-out times due to increased diffusion rates at high temperatures (Bossert and Wilson, 1963). If femoral gland secretions are used as pheromones by *D. dorsalis* in nature, these physical constraints are expected to have influenced the evolution of their chemistry. In particular, low volatility and resistance to degradation at high temperatures are predicted properties of chemical signals utilized by diurnal animals inhabiting desert environments.

METHODS AND MATERIALS

Secretion Collection. Femoral gland secretions were collected from two female and three male *D. dorsalis* captured in the Coachella Valley, Riverside County, California, in April and May 1987. For comparative purposes, secretions were also obtained from one male western fence lizard (*Sceloporus occidentalis;* Iguanidae), one male granite spiny lizard (*S. orcutti*), and one male western whiptail (*Cnemidophorus tigris;* Teiidae) during the same time period. Samples were collected in the field by applying gentle manual pressure around the gland openings of the lizards and removing the secretion plugs with small forceps. Following collection, samples were transported on ice and stored at −15°C. A 5-mg sample of *D. dorsalis* femoral gland secretion was subjected to melting-point analysis.

Chemical Separation. To isolate the nonpolar chemical components, several secretion plugs from both male and female *D. dorsalis* were placed in methylene chloride (CH_2Cl_2), stirred for 1 hr, and stored at room temperature overnight. After filtration of the insoluble components and rotary evaporation of the solvent under reduced pressure, 4.24 mg of CH_2Cl_2-soluble material was recovered and dissolved in 2.5 ml deuterated chloroform ($CDCl_3$). The sample was then stored at −15°C. The insoluble part of the secretion, a white solid representing 79.52% of the total secretion weight, was recovered from the filter, air dried overnight, and stored at −15°C.

Chemical Analyses. A proton nuclear magnetic resonance (NMR) spectrum was obtained on the organic-soluble secretion in $CDCl_3$ on a 360-MHz

NMR spectrometer. Tetramethylsilane was added as a reference compound. Elemental analysis of the CH_2Cl_2-insoluble part of the secretion was carried out to determine the percent carbon, nitrogen, hydrogen, and sulfur present. To examine this part of the secretion in more detail, CH_2Cl_2-extracted secretion plugs from five *D. dorsalis,* one *S. occidentalis,* one *S. orcutti,* and one *C. tigris* were dissolved in 8 M urea to a final concentration of 1 $\mu g/\mu l$ by heating them in a 100°C water bath with intermittent stirring. Upon dissolving, 50 μl of each sample was loaded onto a 12% nonreducing SDS polyacrylamide gel to determine the apparent molecular weight and relative concentration of protein components present (Laemmli, 1970). Molecular weight standards (Biorad, Richmond, California) were also run on the gel. Bromphenol blue was used as the tracking dye. The gel was run at 13 mA for approximately 30 min until the samples had exited the stacker, and then run at a constant 80 V until the sample front had reached the end of the gel approximately 3.5 hr later. Following electrophoresis, the gel was stained with 0.1% Coomassie blue in 50% methanol–10% acetic acid for 20 min, then destained overnight in a 50% methanol–10% acetic acid solution.

Spectroscopic Analyses. Reflectance spectra for fresh whole *D. dorsalis* femoral gland secretion were measured with a LI-COR LI-1800 spectroradiometer and calibrated fiberoptic probe with an acceptance angle of less than 10%. The sample was illuminated with an Oriel model 68735 lamp and reflectance measurements were taken between 300 and 800 nm. To examine the fluorescence of the secretion, the light source was outfitted with a Kodak 18-A filter, which only passes light below 400 nm (transmittance spectrum given in Eastman Kodak Publication B-3, 1981). In order to standardize for the varying intensity of the light source and differential sensitivity of the spectroradiometer at different wavelengths, a measurement was also taken of a polished aluminum standard which reflects nearly 100% of incident light.

RESULTS

Chemical analyses demonstrated that femoral gland secretions of *D. dorsalis* are composed of roughly 80% protein and 20% lipid material by weight and indicated that the secretion begins to decompose when subjected to temperatures of 225°C. It does not melt, even at temperatures in excess of 250°C. Proton NMR spectroscopy of the CH_2CL_2-soluble components of the secretion (Figure 1) reveals peaks exhibiting chemical shifts consistent with the presence of lipids, possibly including triglycerides and sterols (Bhacca and Williams, 1964; Gunstone, 1967). The chemical shifts observed in the spectrum are probably due to tertiary methyl group protons (0–2 ppm region), methylene protons (1.2 ppm), glyceride methylene (4.2 ppm), and olefinic protons (5.2 ppm).

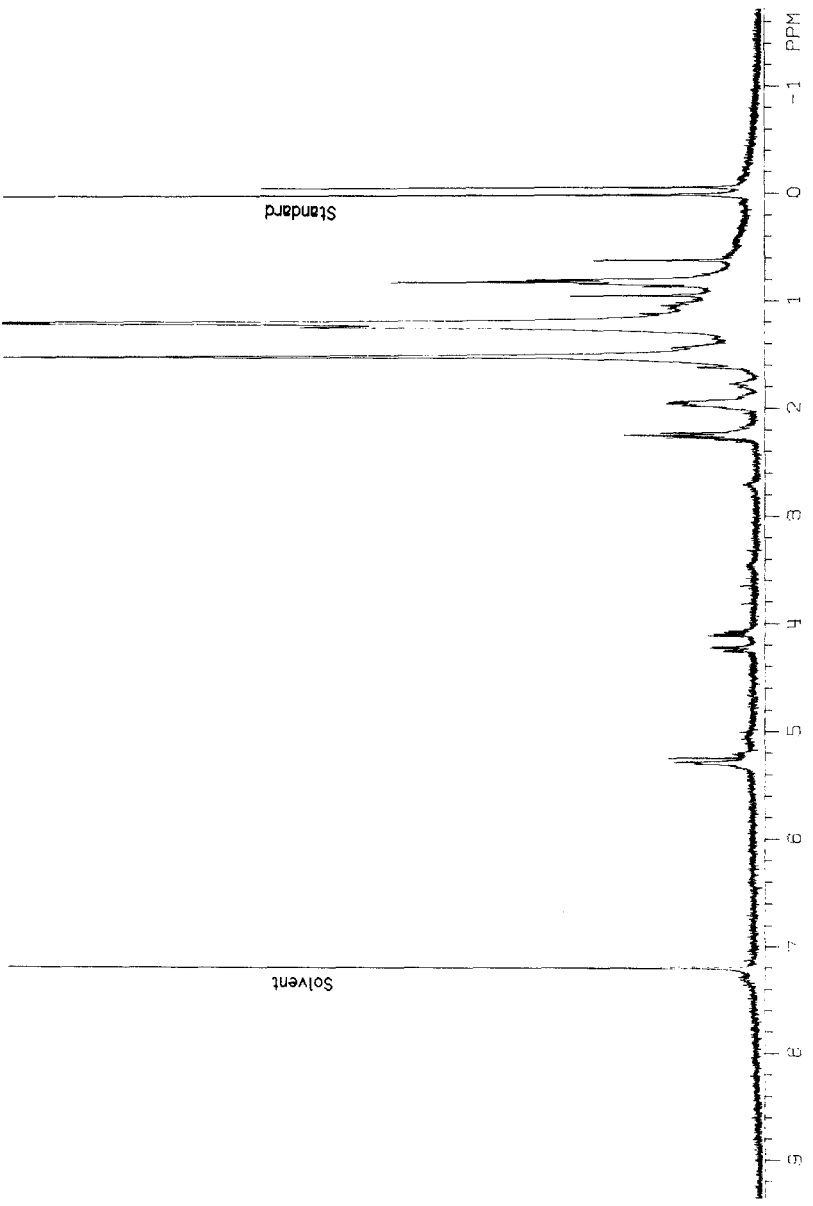

FIG. 1. Fourier transform proton NMR spectrum of femoral gland secretion from the desert iguana, *Dipsosaurus dorsalis*. Solvent is deuterochloroform. Tetramethylsilane (TMS) was added as a reference compound.

Without further supporting data, however, it is difficult to unequivocably identify which lipid classes are present. The peak at 1.5 ppm is probably due to water present in the sample. Elemental analyses of the nonorganic extractable part of the secretion reveals that it is composed of 47.20% carbon, 13.91% nitrogen, 7.09% hydrogen, and 2.82% sulfur. Together with its insolubility, these ratios indicated that this part of the secretion was likely to be proteinaceous in nature. This was supported by spectrophotometry showing a ratio of 220/230 nm absorption equal to 2.98, characteristic of peptide bond linkages.

Results of the 12% SDS polyacrylamide electrophoresis gel are shown in Figure 2. Each of the first five lanes on the gel represents secretions taken from five different individual *D. dorsalis*; the first two lanes are samples from females, the next three are from males. From the staining pattern (Figure 2), the secretion appears to be composed of two major protein components, represented by the darkest bands, at approximately 30,000 and 20,000 daltons, and several smaller components, the majority smaller than 14,000 daltons. While all five *D. dorsalis* possess the same general banding pattern, there are differences among individuals in the lower molecular weight components. Certain components are missing in some individuals, and differences in band intensity indicative of concentration differences are evident. Gels run on these samples which have been stained using the modified silver staining technique of Morrissey (1981) show similar patterns of band intensity (Alberts, unpublished data), indicating that

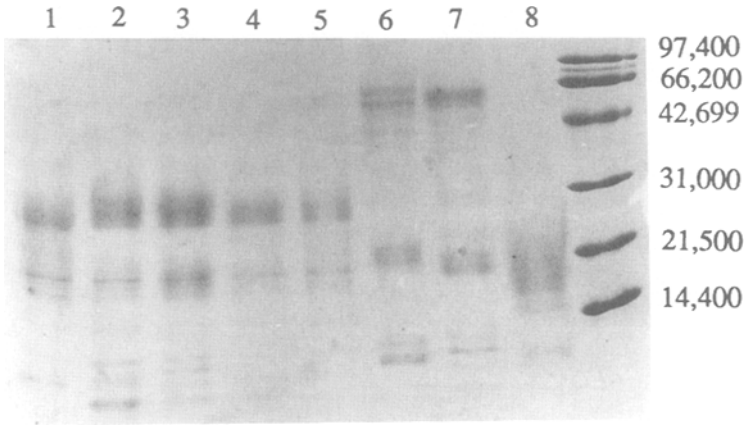

FIG. 2. Results of 12% nonreducing SDS polyacrylamide electrophoresis gel showing banding patterns of femoral gland secretion proteins. Lanes 1 and 2 represent secretions from female *Dipsosaurus dorsalis*, lanes 3–5 are from male *D. dorsalis*, lane 6 is from *Sceloporus occidentalis*, lane 7 is from *S. orcutti*, lane 8 is from *Cnemidophorus tigris*, and a series of molecular weight standards has been run in lane 9.

these concentration differences are not simply a staining artifact. There are no apparent sex differences, but secretions from a greater number of individuals need to be examined before a more definitive conclusion can be reached.

The electrophoretic banding patterns of the other lizards show interspecies difference in the proteins of femoral gland secretions. The two samples from lizard species in the genus *Scelporous* are very similar, and each shows major protein components ranging from 50,000 daltons to less than 14,000 daltons. Three major protein bands are evident in the profile of *S. orcutti.* In *S. occidentalis*, the largest and smallest components are apparent as double bands, indicating that the secretions of this species may contain as many as five different proteins. The banding pattern in *Cnemidophorus tigris* appears to be quite different from those of all three iguanid lizard species, with an indistinct band centered around 20,000 daltons and a faint smaller band below 14,000 daltons.

Figure 3 shows the reflectance spectrum of *D. dorsalis* femoral gland

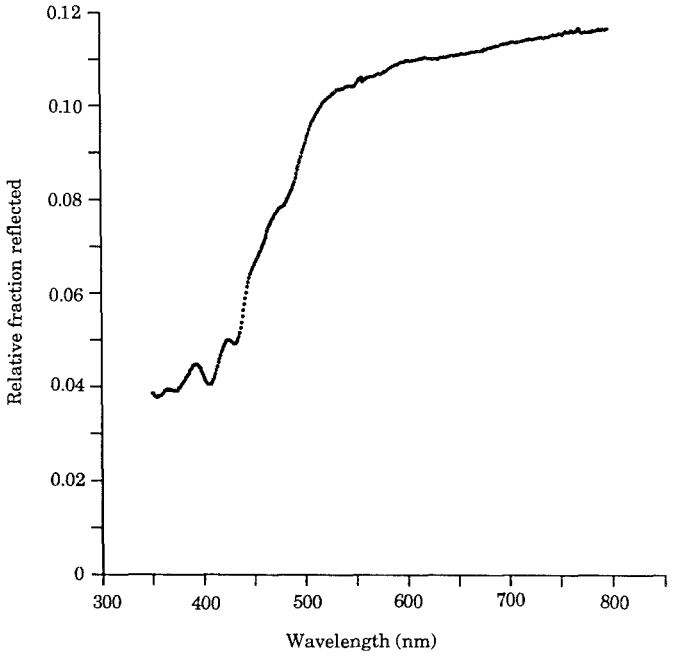

FIG. 3. Reflectance spectrum for *Dipsosaurus dorsalis* femoral gland secretion when illuminated with light between 300 and 800 nm. The curve has been subtracted from that for an aluminum standard, which reflects nearly 100% of incident light, in order to account for the differential output of the light source and sensitivity of the spectroradiometer at different wavelengths. The curve thus represents the fraction of light reflected relative to this standard over the range of wavelengths tested.

secretion when it is illuminated with light ranging from 350 to 800 nm. The curve is plotted relative to an aluminum standard that reflects nearly 100% of incident light. Longwave ultraviolet light between 350 and 400 nm appears to be absorbed rather than reflected by femoral gland secretions, but levels of reflectance rise sharply as the secretions are illuminated with visible light. This steady climb in reflectance above 400 nm is consistent with the whitish appearance of the secretions to the human eye under a visible light source.

The absorption of longwave ultraviolet light by femoral gland secretions results in molecular excitation, producing a visible fluorescence as the excited molecules relax back to their normal lower-energy state. The emission spectrum of fluorescing femoral gland secretion (Figure 4) spans visible light wavelengths from 400 to 600 nm, but its peak corresponds to 500 nm, the yellow-green part

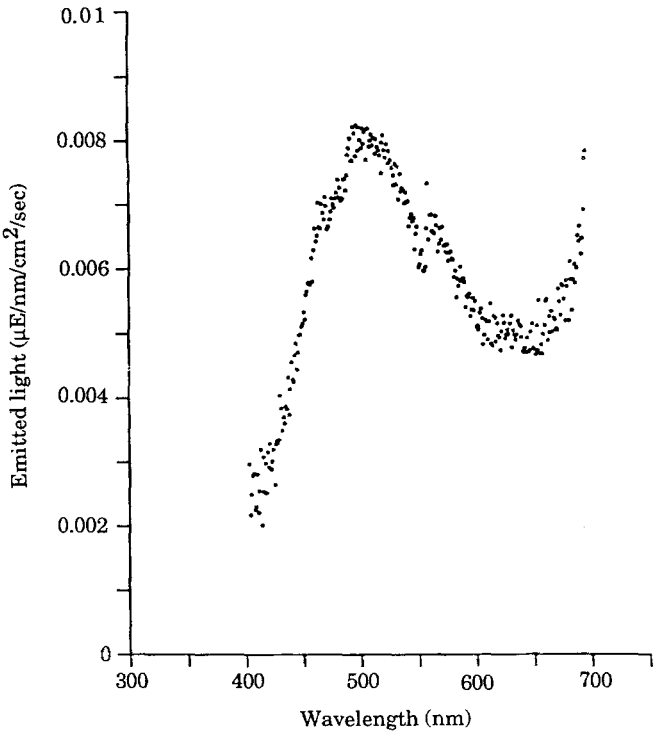

FIG. 4. Emission spectrum showing the production of green light in the 400- to 600-nm range by fluorescing femoral gland secretions of *Dipsosaurus dorsalis* when illuminated with longwave ultraviolet light. The light source has been outfitted with a cutoff filter that only passes light below 400 nm. The visible fluorescence is very weak and does not exceed 0.01 μE/nm/cm^2/sec.

of the visible light spectrum. This is the color perceived by the human eye when femoral gland secretions are viewed under a 365-nm black light in the absence of visible light. This fluorescence is so weak (less than 0.01 microEinstein/nm/cm^2/sec) that it appears to be obscured by ambient sources of visible light and cannot be seen by the human eye under natural light even though human cones have their peak of visual sensitivity at approximately 555 nm (Schenk, 1973).

DISCUSSION

This study has shown that femoral gland secretions of *D. dorsalis* are composed of both lipids and proteins, either or both of which could potentially function as pheromones. Skin lipids that potentially have pheromonal function are known to be widespread in both lizards (Weldon and Bagnall, 1987) and snakes (Burken et al., 1985; Schell and Weldon, 1985). Garstka and Crews (1981) have reported a lipoidal pheromone on the skin of female garter snakes that serves to attract mates. The urodaeal gland pheromones of female broad-headed skinks (*Eumeces laticeps*) have been shown to contain a biologically active neutral lipid fraction containing mono-, di-, and triglycerides, cholesterol, sterol esters, and complex waxy esters (Cooper and Garstka, 1987).

There is abundant evidence for the role of lipids in chemical signalling in a number of vertebrate pheromone systems (Albone, 1984; Hadley, 1985), and theoretically there is good reason to expect that lipids should be widely used as pheromones. The formation by lipids of structural and geometric isomers, and the interchangeability of functional groups on the same basic carbon skeleton of lipids, gives them a high degree of molecular diversity (Hadley, 1985; Cooper and Garstka, 1987). These properties could greatly increase the potential information content of a pheromone (Wilson and Bossert, 1963) without significantly increasing the energetic costs of its biosynthesis.

The protein components of femoral gland secretions, either alone or chemically bound to lipids, could potentially have communicative function as well. While it is possible that femoral gland proteins function only as a matrix to hold lipids, many lizards have specialized chemosensory structures and behavior that could facilitate detection of the proteins themselves. Most lizards possess a vomeronasal organ (Burghardt, 1970; Parsons, 1970) that mediates the perception of molecules brought in on the tongue. The majority of substances that have been shown to stimulate the vomeronasal system are pheromones of relatively high molecular weight including proteins (see Halpern, 1987, for review). The tongue-touching behavior of *D. dorsalis* has been well documented in both the field and the laboratory (Pedersen, 1988; Krekorian, 1989) and is potentially capable of delivering large nonvolatile molecules such as femoral gland proteins to the vomeronasal organ. It is also possible that femoral

gland proteins could be detected by taste receptors on the tongue. Numerous taste buds occur on the tongue tips of iguanid lizards, indicating that tongue-touching behavior could potentially stimulate both the gustatory and the vomeronasal system of lizards (Schwenk, 1985). Beidler (1977) reviews some of the evidence for the detection of proteins based on taste and proposes that although proteins probably require a high degree of stereospecificity in their reception, it is quite possible they could be utilized as contact pheromones in some species.

If *D. dorsalis* are capable of detecting femoral gland proteins via either taste or vomeronasal function, then the individual differences in protein secretion chemistry demonstrated here may be relevant in social communication. Fresh secretion samples collected from the same individual in different months of the year produce identical banding patterns on protein electrophoresis gels (Alberts, unpublished), indicating that these differences are consistent over time. Femoral gland proteins, therefore, could potentially function in individual recognition. Glinski and Krekorian (1985) showed that male *D. dorsalis* are capable of distinguishing familiar from unfamiliar conspecifics, and they hypothesized that chemical cues may play some role in this discriminatory ability. During the breeding season, proteins in the femoral gland secretions of adult lizards are potential candidates for this information.

Interspecific differences in femoral gland proteins appear to be quite striking in the few species studied here and may have ecological relevance in the recognition of heterospecifics for lizards that can detect them. The high degree of correspondence in the major proteins of the two closely related *Sceloporus* species, and the distinctiveness of the banding pattern of the single teiid lizard, *Cnemidophorus tigris*, compared to those of the iguanid lizards, indicates variation along phylogenetic lines. Oldak (1976) noted such strong phylogenetic associations for scent gland lipids in snakes that he proposed secretion chemistry be used as a tool to help evaluate evolutionary relationships between species. The relative roles of phylogeny and ecology in determining the chemistry of femoral gland proteins cannot be adequately evaluated until comparative studies of secretions have been done.

Large proteins have essentially zero vapor pressure, indicating that the bulk of *D. dorsalis* femoral gland secretion is of low volatility. Behavioral tests with *D. dorsalis* demonstrate that the secretions probably have to be contacted directly for information to be obtained from them (Alberts, 1989). This is consistent with results Cooper and Vitt (1986) have reported for the skin and cloacal pheromones of skinks of the genus *Eumeces*. The melting point analyses, which showed that femoral gland secretions retain their durability even at high temperatures, together with the low volatility of these secretions, indicates that these chemical signals will remain active in a desert environment for some time after their deposition. This may be very important for a species like *D. dorsalis*,

which is known to inhabit fairly large home ranges relative to its body size (Krekorian, 1976) and which may not be physically capable of investigating widely dispersed secretion deposits immediately after their release into the environment.

The strong absorption of longwave ultraviolet light by femoral gland secretions may function as a visual cue in the localization of these low volatility secretions in the environment. Behavioral tests with *D. dorsalis* suggest that they are visually sensitive to ultraviolet light in the 365- to 400-nm range, and provision of an ultraviolet light source signficantly increases their ability to locate femoral gland deposits (Alberts, 1989). To these lizards, ultraviolet-absorbing femoral gland secretions may contrast with the sandy substrate in their natural habitat, which is known to be highly reflective of ultraviolet radiation (Frohlich, 1976; Koller, 1985). Thus, absorption of ultraviolet light by femoral gland secretions could act as a long-range visual cue allowing initial localization of low-volatility femoral gland deposits, which are then approached and investigated at close range for more fine-tuned chemosensory information.

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SEX RECOGNITION IN THE LEOPARD GECKO, *Eublepharis macularius* (SAURIA: GEKKONIDAE) Possible Mediation By Skin-Derived Semiochemicals

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Abstract—Male leopard geckoes, *Eublepharis macularius*, rely on skin-derived semiochemicals to determine the sex of conspecifics. Males respond to other males with agonistic behavior while females elicit courtship behavior from males. While females were shedding, males responded to them with agonistic behavior. The same females were courted both before and after shedding. An initial survey of hexane-extracted skin lipids from male and female geckoes revealed fatty acids common to both sexes. Several steroid analogs of cholesterol were unique to males while long-chain methyl ketones were unique to females. Results are discussed in the context of skin lipids serving as pheromones in reptiles.

Key Words—Sex recognition, Gekkonidae, gecko, semiochemicals, sex behavior, agonistic behavior, skin lipids, fatty acids, steroids, methyl ketones.

INTRODUCTION

Sex recognition in lizards is thought to rely primarily on two sensory modalities: vision and chemoreception. Only one of these is of primary importance within a taxon, however (Camp, 1923). For example, gekkonids have long been recognized as utilizing chemical communication in many aspects of their social behaviors (Evans, 1961; Carpenter and Ferguson, 1977), but no experimental studies have been conducted on these lizards.

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Greenberg (1943) observed and quantified social behavior of a gekkonid under laboratory conditions. He proposed two primary mechanisms of sex recognition in the banded gecko, *Coleonyx brevis*: visual displays of stereotyped behaviors and chemoreception of skin-derived chemicals. Greenberg observed that sex recognition in the gecko was not acquired at a distance and close contact was essential before sex recognition occurred. He noted that male *Coleonyx* readily distinguished other males from females and responded accordingly with agonistic or fighting behavior to the former and courtship to the latter. Greenberg hypothesized that females might be identified as mating partners through chemical stimuli. To test this hypothesis, he devised an ingenious experiment. In the majority of courtship displays observed, the male approached from the rear and took a grip on the female's tail. Females were courted further while males were ignored or attacked. Greenberg anesthetized four male and four female geckoes and surgically exchanged their tails. Courting males responded by courting the males with the female's tails. Conversely, when a courting male gripped the male tail of the female, these females were not courted but attacked. He concluded that male *Coleonyx* respond to a chemical stimulus emanating from the skin, reinforcing behavioral cues such as fighting and receptivity to courtship.

The present study continues these investigations of chemical communication in the leopard gecko, *Eublepharis macularius*, a gecko with courtship and agonistic behaviors similar to those of *Coleonyx* (see Gutzke and Crews, 1988). Unlike Greenberg's study, the present study investigates the role of chemical communication in geckoes by observing untreated, intact animals. Based on studies involving other reptiles (Andren, 1982; Radcliffe and Murphy, 1983), we hypothesized that during shedding, those chemical cues contained in or on the female's skin would be temporarily unavailable or in such low concentrations as to be undetectable by males. If skin-derived chemical cues are the primary means by which males distinguish gender, one might expect males to behave differently toward shedding males and females as opposed to the same individuals both before and after shedding. In order to test this hypothesis we observed pairs of geckoes (male–male and male–female) and recorded their behavioral interactions at three distinct periods: preshedding, shedding, and postshedding. Essential in this regard, unlike Greenberg's study, is the fact that in the present study, the male and female stimulus animals were not anesthetized and thus were free to display normal behaviors to the male with which they were paired.

Because Greenberg and others had hypothesized that sex recognition in geckoes is mediated by semiochemicals sequestered on the skin of both males and females, an initial investigation was undertaken to isolate and chemically characterize the skin surface lipids that may serve as semiochemicals in this

lizard. The skin lipids were analyzed by gas chromatography–mass spectrometry (GC-MS).

METHODS AND MATERIALS

Description of Behaviors Measured. Mating behavior in the leopard gecko begins with the male rapidly vibrating his tail and approaching the female slowly. The male then touches the female and begins to lick her tail. He then grips and shakes the female's tail. The biting involved is gentle and does not result in any discernible wounds. The male then shifts his grip to the female's back, neck or head, simultaneously moving his body parallel to hers. If the female is receptive she will raise her tail and allow the male to appose his cloaca to hers and intromission soon follows. A nonreceptive female will terminate courtship by fleeing from the male or biting him (Gutzke and Crews, 1988).

Agonistic or fighting behavior is readily discernible from courtship behavior in the leopard gecko. In the fight pattern, the male raises high off the ground, fully extending his limbs and arching his back. Intense aggression is characterized by the male swelling his throat, followed by short dashes at his opponent and quick, vigorous bites. These bites frequently lacerate the skin and sometimes seriously damage it. Thus, the body posture of the male and the intensity of his bites unequivocally characterize the difference between courtship behavior and agonistic behavior.

Behavior Experiments. Subjects used in this research were sexually mature. Their care and maintenance have been described elsewhere (Bull, 1987). Ten male geckoes were randomly assigned as pairs with 10 female geckoes. The females were then presented to the males in the latter's home cage at three distinct times as determined by the stage in the female's shedding cycle. The preshedding state was defined as at least five days prior to clouding of the skin. Shedding was indicated as the time when the skin was opaque but still intact over the new skin underneath. Finally, postshedding was defined as one day after the complete loss of the old skin. Females were presented to the males once during each of these periods. The order, in terms of shedding condition, in which the females were presented to the male was systematically varied.

The test periods consisted of the female being introduced into the male's cage for 10 min or until the male courted and mounted the female or attacked and drove off the female. As mating may alter subsequent breeding behavior, each trial was concluded before mating or damage was inflicted on the female. Five male geckoes were paired with another five males in a similar fashion.

Isolation and Identification of Skin Lipids. Analytical grade hexane was obtained from Fisher Scientific. Hexane was purified by stirring over H_2SO_4

overnight, decanting and stirring over K_2CO_3 for 4 hr. The solution was filtered, dried over $MgSO_4$ for 1 hr, and redistilled before use.

The five adult male and nine female geckoes were killed with an overdose of Brevital sodium and placed dorsal side down in a separate 500-ml glass beaker with 5–10 ml of hexane. Care was taken that the head and cloaca did not touch the solvent in order to prevent contamination of the wash with body fluids. Each beaker was covered with aluminum foil and sealed with parafilm and left overnight at room temperature. The bodies were removed and the solution filtered through a sintered glass funnel to remove particulate matter and inorganics present on the skin. The extracted lipids were pooled and the solvents removed on a rotavaporator at 50°C. The resulting viscous semisolid mixture was redissolved in fresh hexane, transferred into 7.0-ml amber glass vials with Teflon caps, and stored at −20°C.

Capillary gas chromatographic analyses were performed on a Finnigan-MAT 4920 with a combined quadropole mass spectrometer. This system is supported by the INCOS data system and includes a library of over 42,000 spectra. The capillary column was a fused silica 4-m BP1 (0.5 μm film) (Scientific Glass Engineering). Samples were analyzed under electron impact (EI) mode. Helium was used as a carrier gas in this instrument at 12 psi. The conditions of the GC-MS were as follows: injector temperature at 290°C, source at 150°C, detector at 250°C, EM voltage at −2200 volts. The temperature program was set at 60°C for 1 min. ramping to 290°C at 10°C/min. Samples (1 μl/10 mg/ml) were injected by means of a 5-sec splitless injection.

RESULTS

The results of the behavior tests were unambiguous. The test males all courted their respective stimulus females during the preshedding and postshedding phases of the test (Figure 1). However, during the time when the females were shedding, all the males responded to them with aggressive behavior. Indeed, one female was attacked and had to be removed from the experiment. Thus, each male responded to the same female with both courtship behavior and aggressive behavior depending on the shedding stage of the female. No differences in the behavior of the females were detected during the course of this study. Males always exhibited aggressive behavior towards other males irrespective of the shedding stage of the stimulus male.

Identities of components by GC-MS were made by comparison to published spectra and comparison of GC retention time and similarity to synthetic standards. The mass spectral data revealed at least 33 components of the skin lipids of male and female geckoes (Figure 2, Table 1). The majority of the components were shared in common by both males and females. For instance, fatty acids with carbon chain lengths of C_{16} and C_{18} were the major peaks in

Females

Pre-shedding (5 days prior to clouding)		Shedding		Post-shedding (1 day post-shedding)	
N	Results	N	Results	N	Results
9	Males display sexual behavior	10*	Males display aggressive behavior	9	Males display sexual behavior

* One animal was severely injured and was removed from the experiment

FIG. 1. Behavioral responses of male leopard geckoes, paired with conspecific females at three distinct phases of the female's shedding cycle.

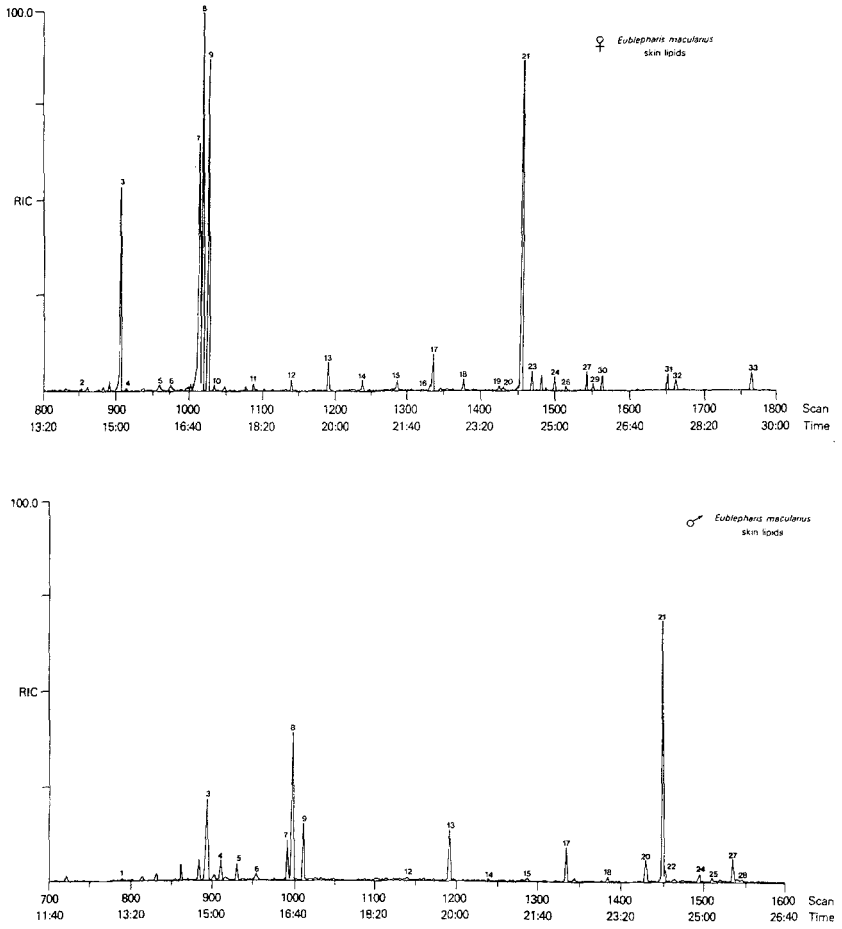

FIG. 2. Gas chromatograms of female and male leopard geckoes. The numbered peaks are identified in Table 1. Retention time is in minutes.

TABLE 1. SKIN LIPID COMPOUNDS FROM HEXANE EXTRACTS OF LEOPARD GECKO

Peak	Mol. Wt.	Elemental formula	Chemical name
1	254	$C_{18}H_{38}$	octadecane
2	268	$C_{19}H_{40}$	nonadecane
3	256	$C_{16}H_{32}O_2$	palmitic acid
4	282	$C_{20}H_{42}$	eicosane
5	270	$C_{17}H_{34}O_2$	heptadecanoic acid
6	296	$C_{21}H_{44}$	heneicosane
7	280	$C_{18}H_{32}O_2$	linoleic acid
8	282	$C_{18}H_{34}O_2$	oleic acid
9	284	$C_{18}H_{36}O_2$	stearic acid
10	310	$C_{22}H_{46}$	docosane
11	324	$C_{23}H_{48}$	tricosane
12	338	$C_{24}H_{50}$	tetracosane
13	352	$C_{25}H_{52}$	pentacosane
14	366	$C_{26}H_{54}$	hexacosane
15	380	$C_{27}H_{56}$	heptacosane
16	394	$C_{28}H_{58}$	octacosane
17	410	$C_{30}H_{50}$	squalene
18	408	$C_{29}H_{60}$	nonacosane
19	422	$C_{30}H_{62}$	triacontane
20	400	$C_{28}H_{48}O$	cholest-5-en-3-methoxy
21	386	$C_{27}H_{46}O$	cholest-5-en-3β-ol
22	388	$C_{27}H_{48}O$	cholestanol
23	436	$C_{31}H_{64}$	hentriacontane
24	400	$C_{28}H_{48}O$	ergost-5-en-3β-ol
25	412	$C_{29}H_{48}O$	stigmasterol
26	450	$C_{32}H_{66}$	dotriacontane
27	414	$C_{29}H_{50}O$	stigmast-5-en-3β-ol
28	416	$C_{29}H_{52}O$	stigmastanol?
29	448	$C_{31}H_{60}O$	hentriacontenone
30	450	$C_{31}H_{62}O$	hentriacontanone
31	476	$C_{33}H_{64}O$	tritriacontenone
32	478	$C_{33}H_{66}O$	tritriacontanone
33	504	$C_{35}H_{68}O$	pentatriacontenone

terms of concentration in both males and females. The other major peak shared in common by both sexes is cholesterol, which is ubiquitous in vertebrate tetrapod epidermis. A minor component shared by both sexes is squalene. Finally, a series of straight-chain hydrocarbons was noted in both sexes at approximately equal levels.

Some differences appear in the skin lipids of higher mass. For example, both sexes have several steroids that are analogs of cholesterol including cam-

pesterol and sitosterol, as well as the methyl ether of cholesterol. However, males also possess cholestanol, stigmasterol, and stigmastanol, which do not appear in the females' skin lipids. Females, on the other hand, possess a series of long-chain saturated and mononunsaturated methyl ketones that are not found in the male chromatograms.

DISCUSSION

The results of this study demonstrate that the behavioral responses of male leopard geckoes toward females differ over the course of the female's shedding cycle. We propose that chemicals in the skin of females normally detected by males become unavailable while the female is shedding. Males responded to these shedding females in the same way that they responded to males. Males attacked other males whether those males were shedding or not. *Eublepharis macularius* does not exhibit obvious sexual dimorphism so perhaps visual cues alone would not be sufficient for males to determine gender. Unlike some geckoes, *Eublepharis* does not use vocalization to any great degree. It seems evident from these data that if a male cannot ascertain a conspecific's gender by means of chemical cues, he behaves toward that individual as if it were a male and attacks it.

These observations and hypotheses are supported by similar behaviors observed in other reptiles. For instance, skin-derived chemical cues sequestered in female garter snake skin elicit courtship behavior from males (Noble, 1937; Crews, 1976; Kubie et al., 1978; Garstka and Crews, 1981; Mason and Crews, 1985, 1986). Additionally, among zoo workers it is almost axiomatic, although not essential, that snakes breed best soon after the female has shed her skin (Radcliffe and Murphy, 1983). These authors state that the frequency with which courtship and copulation follow skin shedding in snakes strongly suggests that there is a sex pheromone associated with the newly shed snake and skin that acts as a releaser of courtship.

Perhaps the closest approximation to the behaviors observed in this study occur in the European adder, *Vipera berus* (Andren, 1982, 1986). The males in this species are aggressive and fight for access to unmated females. Vernal courtship behavior begins only when sexually active males shed their skin. In the adder, males actively tongue-flick chemical cues from shed females and initiate courtship behavior. When a male encounters another shed male, he begins combat behavior. Interestingly, in the adder, if a recently shed male encounters a nonshed male or female, he will ignore them. Andren (1982) concluded that male European adders must obtain chemical information from a conspecific's skin in order to initiate courtship or combat behavior.

Anecdotal observations suggest that diverse reptilian groups use sex pher-

omones, but few aspects of their chemistry are known. Only two studies have investigated the chemical constituents of skin lipids in any lizard, and these did not focus on intraspecific differences between the sexes. Roberts and Lillywhite (1980) isolated skin lipids from the green iguana (*Iguana iguana*) by thin-layer chromatography (TLC). They identified lipids in several classes, including fatty acids, sterol esters, cholesterol, wax esters, and others. The other study was an extensive survey of the shed skins of 23 species of lizards by TLC and demonstrated the great variability in the composition of skin lipids in lizards (Weldon and Bagnall, 1987). These authors conclude that more details are needed on the chemical structures of these lipids, many of which could not to be determined by TLC techniques.

The present study identifies several lipids suggested in previous studies of saurian skin lipids, thus verifying their presence in lizard skin. Cholesterol is a common constituent in all vertebrate skin and seems to be present in all of the reptiles examined thus far. Fatty acids are also commonly found in the skin lipid profiles of vertebrates including reptiles (Ahern and Downing, 1973; Weldon and Bagnall, 1987; Mason et al., 1987). For example, fatty acids have been demonstrated to serve as pheromones in four species of tortoise (*Gopherus*). Rose (1970) identified several fatty acids in the mental glands of male tortoises. He applied either a control solution of solvents or a mixture of fatty acids to the head of a plaster tortoise model. Females responded by approaching the models and head-bobbing, a display indicating courtship. Males responded by ramming the models—clearly an aggressive response.

The presence of straight-chain hydrocarbons in the skin lipids of both male and female geckoes is an interesting finding. Hydrocarbons were identified in the indigo snake, *Drymarchon corais*, but were thought to be contaminants (Ahern and Downing, 1973). However, evidence of hydrocarbons was found in a TLC survey of skin lipids in lizards by Weldon and Bagnall (1987). These authors concluded that the hydrocarbons indicated in their study were not contaminants and that hydrocarbons may occur commonly among squamates. We agree that the hydrocarbons identified in the present study are indeed components of the skin lipid profiles of these lizards. The possible function of these integumental hydrocarbons warrants further study.

Other possible sources of semiochemicals in these reptiles include the sex differences found in the steroids and methyl ketones. Although the steroids identified here are not sex steroids (androgens or estrogens and progestins), they may still impart information about the sex of the individual. Finally, the methyl ketones found in the females' skin lipids are known to occur in female snakes. Ahern and Downing (1973) and Schell and Weldon (1985) identified methyl ketones in the skin lipids of a female indigo snake, *Drymarchon corais*, while Mason (1987) has identified them in the skin lipids of female garter snakes, *Thamnophis sirtalis parietalis*, where they serve as sex attractiveness pheromones (Mason, 1987).

Both Maderson (1986) and Graves et al. (1986) have suggested that reptilian skin lipids have been protoadapted or exapted to serve as semiochemicals or pheromones. It is now widely acknowledged that skin lipids originally evolved in reptiles to serve as a water-retention mechanism. Over the course of evolutionary time, males and females of a given species have acquired the ability to recognize and respond differentially to integumental chemical cues of conspecifics. It has been demonstrated in this study and a previous study (Mason et al., 1987) that clear sex differences exist in skin lipids, which would be a necessary condition for this mechanism of sex recognition to occur.

The results of this work and the previously cited studies pose some questions on the evolution of skin-derived pheromones in reptiles. In the case of sex recognition pheromones, do males of various species of reptile cue in on a set of specific components of the female's skin lipids such as fatty acids, steroids, or methyl ketones? If this is generally the case, as has been demonstrated in a few species, how could such a mechanism evolve?

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RESPONSES BY CORN SNAKES (*Elaphe guttata*) TO CHEMICALS FROM HETEROSPECIFIC SNAKES

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Abstract—Young corn snakes, *Elaphe guttata*, were tested for responses to chemicals from heterospecific snakes. Corn snakes exhibited more tongue-flicks to swabs freshly rubbed against the skin of an ophiophagous kingsnake, *Lampropeltis getulus*, than to blank swabs. Responses to *L. getulus* and a nonophiophagous western plains garter snake, *Thamnophis radix haydeni*, did not differ significantly. Corn snakes exhibited more tongue-flicks to swabs treated with chloroform extracts of the shed skins of *L. getulus*; an ophiophagous eastern coachwhip, *Masticophis flagellum*; and a nonophiophagous gray ratsnake, *Elaphe obsoleta*, than to blank swabs, but they did not discriminate between ophiophagous and nonophiophagous species in every case. Corn snakes, when offered shelters containing bedding from the home cages of a nonophiophagous water snake, *Nerodia erythrogaster*; an occasionally ophiophagous water moccasin, *Agkistrodon piscivorus*; or *L. getulus* and untreated bedding, failed to reside under snake-scented shelters at a rate significantly different from that expected by chance. The responses of corn snakes are compared with those reported for other snakes presented with heterospecific snake chemicals.

Key Words—Corn snake, *Elaphe guttata*, skin chemicals, predator detection.

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INTRODUCTION

Snakes enact a variety of defensive behaviors, including biting, tail display, and cloacal discharge, when confronted directly by predators (Greene, 1987). Avoidance of predators also should contribute to survivorship, but for this to occur mechanisms to recognize enemies at a distance must exist.

Many snakes exhibit responses to chemicals from ophiophagous snakes. Rattlesnakes, *Crotalus* spp. (Crotalinae), respond to the kingsnake, *Lampropeltis getulus*, by a posture known as "body bridging," where their trunk is raised off the substrate. Bogert (1941) showed that this behavior, which may obstruct attempts to grasp rattlesnakes, is elicited by chemicals from the dorsal skin of kingsnakes and other species. Since the publication of Bogert's report, other crotalines have been observed body bridging during encounters with snake predators (Carpenter and Gillingham, 1975) or during presentations of chemicals from them (Weldon and Burghardt, 1979; Marchisin, 1980).

The involvement of chemoreception in the detection of ophiophagous snakes by colubrid snakes (Colubridae), the most speciose snake family, has been examined in a few studies. Garter snakes, *Thamnophis* spp., presented with swabs rubbed against the skin of heterospecific snakes or with an airstream directed over them, exhibited more tongue-flicks to chemicals from ophiophagous snakes than to those from some generally nonophiophagous species (Weldon, 1982). Young pine snakes, *Pituophis melanoleucus*, orient toward trails deposited by conspecifics but not to those of kingsnakes, suggesting a mechanism by which pine snakes avoid these predators (Burger, 1989). The hognose snake, *Heterodon platyrhinos*, on the other hand, failed to exhibit defensive responses or changes in tongue-flicking rate to filter papers containing kingsnake odors (Durham, 1980).

No systematic tests have been conducted on the behavioral defenses of ratsnakes, *Elaphe* spp., but rapid flight from kingsnakes (Weldon, 1982) and the delivery of body blows toward these predators (Marchisin, 1980) have been observed. Field studies involving wholesale collections of snakes and analyses of their stomach contents provide information consistent with the hypothesis that *Elaphe* spp. possess mechanisms that reduce snake predation. The results of these investigations indicate that a variety of snake species are taken as prey, but that *Elaphe* spp. are absent (Uhler et al., 1939; Hamilton and Pollack, 1955, 1956; Brown, 1979) or rare (Clark, 1949) in the stomachs of ophiophagous snakes, even though they occurred in the areas surveyed.

We report here the results of tests of the corn snake, *Elaphe guttata* (Colubridae), with fresh and chloroform-extractable skin chemicals from heterospecific snakes, including those from ophiophagous species. The responses of

corn snakes to heterospecific snake skin chemicals are compared with those of other snakes.

METHODS AND MATERIALS

Swab Presentations. In experiment 1, nine corn snakes [$\bar{X}$ snout–vent length (SVL) = 32 cm], hatched from eggs laid by a female captured in Blount County, Tennessee, were tested for responses to swabs freshly rubbed against the skins of stimulus snakes. Snakes were individually housed in 31 × 16 × 8-cm clear plastic boxes with lids; subjects in this and all other experiments were maintained and tested at 25–27°C. Snakes were 5 weeks old at the time of testing. Each ate a small mouse five days before testing began.

Snakes were presented in their home cages with cotton swabs laden with skin chemicals from adults of a kingsnake and a western plains garter snake, *Thamnophis radix haydeni*; only the former is ophiophagous. Swabs were dipped into methylene chloride (CH_2Cl_2) and rubbed against the dorsal surface of a stimulus snake. Control swabs were dipped into CH_2Cl_2.

Swabs were allowed to air-dry for 15 min before being held in front of a subject's snout for 2 min while tongue-flicks were counted. Each snake was presented with a different condition over three consecutive days in a Latin-square design. Testing was done between 1500 and 1600 hr.

In experiment 2, eleven corn snakes ($\bar{X}$ SVL = 29 cm), hatched from eggs laid by a female captured in Aiken county, South Carolina, were tested for responses to swabs treated with chloroform ($CHCl_3$) extracts of shed snake skins. Snakes were individually housed and tested in 9.5 × 18-cm cylindrical glass containers, the bottom of which was lined with a paper towel. Subjects had access to water ad libitum. They were 7 weeks old at the time of testing. Each ate a small mouse five days before testing began.

A shed skin (1.8 g) of an adult kingsnake was placed into 400 ml of $CHCl_3$ and heated (40°C) for 2 hr. The $CHCl_3$ was poured off and the extraction procedure was repeated. The combined extracts were placed on a rotary evaporator to remove the solvent. The residue was redissolved in $CHCl_3$, transferred to a vial, and the solvent was removed under nitrogen. After drying in vacuo, the weight of the residue was 10 mg. A 5 mg/ml $CHCl_3$ solution of snake skin extract was prepared; swabs treated with shed snake skin solutions have elicited heightened tongue-flicking and predatory attack in kingsnakes (Weldon and Schell, 1984).

Swabs were dipped into $CHCl_3$ or a $CHCl_3$ skin extract and allowed to air-dry for 20 min. Swabs were then presented as described above while tongue-

flicks were counted. Each snake was presented in its home cage with a control or a snake-scented swab once a day. The alternate condition was presented on the next day. Swabs were stored at −70°C between tests. All tests were run between 1700 and 1900 hr.

In experiment 3, eleven corn snakes used in the previous experiment were tested for responses to swabs treated with shed skin extracts from several snake species. Snakes were maintained and tested in their home cages as described for experiment 2, except that water dishes were removed from their containers 12 hr before testing. Subjects were 11 weeks old at the time of testing. Each ate a small mouse four days before testing began.

Shed skins were obtained from adults of a gray ratsnake, a kingsnake, and an eastern coachwhip, *Masticophis flagellum*; the latter two species are routinely ophiophagous. The skins (7.3, 4.7, and 2.4 g, respectively) were extracted in $CHCl_3$. The solvent was removed by rotoevaporation. Residues of 10, 17, and 10 mg, respectively, were obtained. A 5 mg/ml $CHCl_3$ solution was prepared from each.

Swabs were dipped into each solution and allowed at least 20 min to air-dry before being presented to the subjects. Control swabs were dipped into $CHCl_3$. They were presented as described above in a Latin-square design over one day from 1000 to 1600 hr, with at least 1 hr between presentations.

Substrate Presentations. Fourteen corn snakes ($\bar{X}$ SVL = 40 cm), hatched from eggs laid by females from Aiken and Barnwell counties, South Carolina, were tested for responses to substrate-borne chemicals from heterospecific snakes. Snakes were individually housed in 40 × 27 × 17-cm plastic cages. Subjects were 1 year old at the time of testing. Each ate a mouse one week before testing began.

The stimulus snakes were adults of a yellow-bellied water snake, *Nerodia erythrogaster*; a water moccasin, *Agkistrodon piscivorus*; and a kingsnake. The latter species is routinely ophiophagous, *A. piscivorus* is occasionally so. Snakes were maintained in 40 × 27 × 17-cm plastic cages lined with corncob bedding, which was collected after three days from each snake's home cage.

Snakes were tested over 21 days in either of two 0.8-m-high circular tanks (diameter = 270 cm) lined with wrapping paper. Eight 30 × 30-cm ceramic tile refuges were elevated 5 cm above the substrate on blocks of wood evenly spaced around the perimeter of each arena. Approximately 250 ml of bedding from a stimulus snake was placed under two refuges situated opposite one another; untreated bedding was placed under the other refuges. The position of the refuges containing snake-scented bedding was randomized.

Each *E. guttata* was placed individually in the center of a tank at 1600 hr and allowed to move freely. The subject's location was noted at 0900 and 1600 hr the following day, and at 0900 hr on the third day. A snake was scored

present at a site if it was observed on two of three occasions under the same type of refuge. The arena was washed with ethanol, lined with fresh wrapping paper, and equipped with new tiles and wood blocks after each test.

RESULTS

The Kruskal-Wallis test applied to data from experiment 1 (Table 1) detected overall significant differences in tongue-flick scores ($H = 8.9$; $P \leq 0.05$). The STP aposteriori test (Sokal and Rohlf, 1981) detected significant differences between tongue-flick scores to kingsnake-treated and blank swabs ($P \leq 0.05$). No other significant differences among treatment groups were detected.

In experiment 2, snakes exhibited mean tongue-flick scores ($\pm$ 1 standard deviation) of 68.5 $\pm$ 27.1 and 44.5 $\pm$ 19.9 during 2 min to the kingsnake- and solvent-treated swabs, respectively. The Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) detected a significant difference between these scores ($P \leq 0.05$).

The Kruskal-Wallis test applied to data from experiment 3 (Table 2) detected overall significant differences in tongue-flick scores ($H = 20.4$; $P \leq 0.05$). The STP a posteriori test detected significant differences between the tongue-flick scores to the blank swabs and each of the three snake scents, between scores to kingsnake and eastern coachwhip scents, and between scores to eastern coachwhip and corn snake scents ($P \leq 0.05$ in all cases). No significant difference was detected in the tongue-flick scores to kingsnake and corn snake scents.

Snakes in the substrate tests were scored four, three, and two times under the shelters containing the bedding from water snake, water moccasin, and kingsnake, respectively. The chi-square test failed to indicate that snakes resided under snake-scented shelters at rates significantly different from that expected by chance ($P > 0.05$).

TABLE 1. MEAN TONGUE-FLICKS ($\pm$1 SD) BY NINE *Elaphe guttata* FOR 2 MIN TO BLANK SWABS AND SWABS FRESHLY RUBBED AGAINST DORSAL SKIN OF STIMULUS SNAKES

Lampropeltis getulus	*Thamnophis radix haydeni*	Blank
73.5 + 29.7	54.4 + 15.9	44.0 + 21.8

TABLE 2. MEAN TONGUE-FLICKS (±1 SD) BY 11 *Elaphe guttata* FOR 2 MIN TO BLANK SWABS AND SWABS TREATED WITH CHLOROFORM EXTRACTS OF HETEROSPECIFIC SHED SNAKE SKINS

Elaphe obsoleta	*Lampropeltis getulus*	*Masticophis flagellum*	Blank
98.5 ± 33.6	105.0 ± 24.8	68.0 ± 31.4	40.5 ± 30.1

DISCUSSION

Anecdotal accounts indicate that rattlesnakes and other crotalines exhibit less tongue-flicking during confrontations with kingsnakes or while body bridging (Carpenter and Gillingham, 1975; Marchisin, 1980). Chiszar et al. (1978) demonstrated that water moccasins exhibit fewer tongue-flicks in a tank previously occupied by a kingsnake than in a clean tank or in one that had contained a nonophiophagous hognose snake; a similar trend with rattlesnakes however, was not significant. Marchisin (1980) suggested that the infrequent and reduced tongue-flicking to ophiophagous snakes prevents a predator's attention from being drawn to a crotaline's head.

Tests of garter snakes with both swab-laden and airborne chemicals indicated that they detect heterospecific snakes and that they tongue-flick more to chemicals from ophiophagous species (Weldon, 1982). Weldon (unpublished) found that garter snakes also exhibit more tongue-flicks in glass tanks that previously had housed a kingsnake than in ones that either had contained a ratsnake or had been misted with distilled water. Several subjects, in the kingsnake-conditioned tanks only, emitted slow-motion tongue-flicks; this behavior also is exhibited by garter snakes in response to human molestation (Gove and Burghardt, 1983) and was hypothesized by Gove (1979), for snakes in general, to have a defensive (warning) function.

The results of our experiment 1 indicate that corn snakes tongue-flick more to swabs freshly rubbed against the integument of a kingsnake than to blank swabs, but significant differences in tongue-flick scores were detected for neither garter snake vs. blank nor garter snake vs. kingsnake conditions. In experiment 2, corn snakes responded more to chloroform-extracted chemicals from a kingsnake shed skin than to blank swabs. Similarly, the results of experiment 3 indicate that corn snakes detect chemicals extracted from the skins of a kingsnake, an eastern coachwhip, and a gray ratsnake. The apparent failure of our subjects to distinguish (as indicated by tongue-flick scores) between the kingsnake and the garter snake in experiment 1, and between the kingsnake and the ratsnake in experiment 3, however, is not consistent with the hypothesis that

corn snakes distinguish between chemicals from ophiophagous and non-ophiophagous snakes.

Corn snakes failed to reside under snake-scented shelters in our substrate experiment at a rate significantly different from that expected by chance. The concentrations of chemicals contained on the bedding material we used may have been insufficient for our subjects to detect; it is unclear whether naturally occurring deposits from snakes in the field could provide more potent stimuli. In an analogous test, Parker (1978) found that several crotaline snakes, including species of *Agkistrodon*, *Crotalus*, and *Sistrurus*, failed to avoid the side of an arena that had contained a kingsnake. Using a Y maze, Burger (1988) observed that pine snakes follow trails deposited by conspecifics but not those left by kingsnakes. Trail-following experiments need to be performed comparing the responses to ophiophagous and nonophiophagous stimulus snakes. Tests of this sort may indicate responses that can be related more directly to features likely to confer an advantage vis-à-vis predators in nature.

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A COMPARATIVE ANALYSIS OF SCORING METHODS FOR CHEMICAL DISCRIMINATION OF PREY BY SQUAMATE REPTILES

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Abstract—In studies of squamate responses to prey chemicals presented on cotton-tipped applicators, investigators typically record several responses, each of which gives only part of the overall picture. The tongue-flick/attack score (TFAS) is a widely used composite measure of response strength that accounts for attack, its latency, and number of tongue-flicks. We present data and analyses on these variables and investigate the utility of several other possible response variables. It is concluded, for both practical and theoretical reasons, that TFAS and two modifications of it are the best measures of response strength. Uses and statistical analyses of TFAS and variables derived from it are discussed. It is recommended that information on tongue-flick rate, number of individuals attacking, and latency to attack be presented.

Key Words—Reptilia, tongue-flicks, broad-headed skink, *Eumeces laticeps*, garter snake, *Thamnophis sirtalis*.

INTRODUCTION

Many lizards and snakes respond to chemicals presented on cotton-tipped applicators (swabs) by tongue-flicking and, in some cases, by biting the swab, especially if it bears chemical stimuli from preferred prey. Measurements of tongue-flick rate and latency to bite have been combined by Burghardt (1967, 1970) to

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provide a single index of overall response strength to a particular stimulus: the tongue-flick/attack score (TFAS). Some investigators have used different measures (e.g., Halpern and Frumin, 1979; Arnold, 1981), but the TFAS has become a standard and highly successful tool for studying the ability of snakes and lizards to discriminate prey chemicals from odorless controls and biologically irrelevant but odorous controls presented on swabs.

Various aspects of data obtained in such studies often are not presented or analyzed. Among these are tongue-flick rate, latency to bite, and number of individuals biting. In this study, results on raw tongue-flick rate and latency to bite are compared to each other and to the TFAS. The greatest advantage of TFAS as a measure of response strength is that it incorporates both tongue-flicks and latency in a single index. Although TFAS is a somewhat arbitrary measure of response strength, its possible strengths and weaknesses have not received much attention. Burghardt (1970) developed the TFAS based on the assumptions that an attack indicates a stronger predatory response than any number of tongue-flicks and that latency decreases with increasing stimulus strength. He selected maximum number of tongue-flicks by any individual as an arbitrary but reasonable base unit for TFAS for individuals that did attack. He scaled the relationship between number of tongue-flicks and latency by adding one TFAS unit per second for every second of a projected 60-sec testing interval remaining at the time biting occurred. Thus, if the subject did not attack, TFAS was given by total number of tongue-flicks in 60 sec. If an animal bit, its TFAS was the base unit + (60 − latency). Similar results were obtained using 30-sec trials and adjusting the contribution of latency accordingly (Burghardt, 1975).

A preliminary comparison of TFAS, biting, and latency by Burghardt (1970) showed that results based on TFAS were similar to those for number of individuals biting in *Thamnophis r. radix*, but the agreement was not complete. Results on latency differed somewhat from those for TFAS. However, because all snakes responded to worm extract, these comparisons may not be directly relevant to chemical discrimination experiments in which individuals respond to several different stimuli.

The goals of this study were (1) to examine data on tongue-flick rate (TFR), latency to bite, and number of bites to determine whether information might be lost by amalgamating tongue-flicking and latency data in the TFAS; (2) to determine relative merits of other potential variables; (3) to consider whether the TFAS is a conceptually optimal variable for analysis; and (4) to suggest possible improvements in response measures and their analysis. A possible alternative method of calculating response strength that accounts for both tongue-flicks emitted and latency to bite was investigated. In addition, a modified method of calculating TFAS for repeated-measures designs was considered.

METHODS AND MATERIALS

Data from a study of prey chemical discrimination in the broad-headed skink, *Eumeces laticeps* (Cooper and Vitt, 1989), were used to compare results based on various response measures. Each of 12 lizards was tested with deionized water, a pungency control (cologne), and chemicals from cricket integument. One stimulus per day was presented in a counterbalanced sequence on a cotton-tipped swab. Numbers of tongue-flicks directed toward swabs were counted for 60 sec beginning with the first tongue-flick or until the lizard bit the applicator (if this occurred within 60 sec). Latency to bite also was recorded.

The primary data analyzed were TFR, latency, and tongue-flick/attack scores (TFAS) calculated as in Burghardt (1970). If a lizard did not bite the swab, its TFAS for that condition was the number of tongue-flicks emitted in the 60-sec interval. If it did bite, the TFAS was (60 sec minus latency to bite in seconds) plus the maximum number of tongue flicks emitted by any lizard in any of the three conditions. The TFAS heavily weights biting, assuming that it indicates a stronger predatory response than any number of tongue-flicks.

An additional analysis was conducted involving a modification of the traditional TFAS for use with experimental designs in which a single individual responds to stimuli in multiple conditions. TFAS(R) was calculated by adding the greatest number of tongue-flicks for any stimulus for each individual (rather than for all individuals) to the latency component. That is, for each individual, i, tested for response to several stimuli,

$$TFAS(R)_i = TF_{\max_i} + (TL - \text{latency}_i)$$

In this notation the traditional calculation is

$$TFAS_i = TF_{\max} + (TL - \text{latency}_i)$$

where $TF_{\max}$ is the largest number of tongue-flicks emitted in any trial in the entire study, TL is trial length in seconds in the absence of attack, and $TF_{\max_i}$ is the maximum number of tongue-flicks emitted by individual i in any trial.

Another method of scaling responses was investigated. Instead of giving heavy weight to bites, the tongue-flick rate prior to biting was projected through the entire minute. In this projected tongue-flick rate (PTFR), the response measure was

$$PTFR = \text{tongue flicks}^* \frac{TL}{\text{latency}}$$

This measure was used by Arnold (1978) as a primary index of response to prey chemicals and by Halpern and Frumin (1979) as part of a response measure

including effects of biting and latency. PTFR may be interpreted in two ways. First, it may indicate the number of tongue-flicks that would have occurred had the subject not bitten. A potential problem with interpreting PTFR in terms of tongue-flick rates is the assumption that the rate is constant throughout the test. Because no adequate data on response to prey chemicals were available, this assumption was examined using data from pheromone studies for responses to deionized water, cologne (English Leather), aqueous swabs of male cloacal epithelia, and aqueous swabs of female cloacal epithelia (Cooper and Vitt, 1984, 1986b). Tongue-flicks emitted by adult male *E. laticeps* during the first 20 sec were compared to total tongue-flicks in the final 40 sec divided by two. These data were subjected to *t* tests for correlated samples. In the second interpretation, PTFR is considered an arbitrary index of response strength that combines effects of tongue-flicks and biting. In the latter case, PTFR heavily weights early biting if tongue-flick rates are highest early in the trials. Thus, possible effects of variation in response rate among stimuli are important for both interpretations of PTFR.

After data from the prey chemical discrimination experiment were tested for homogeneity of variance with Hartley's test, a single-factor ANOVA for a repeated-measures design was conducted (Winer, 1962) if variances were homogeneous. Individual comparisons among treatment means were made using Newman-Keuls tests (Winer, 1962). The alpha value was 0.05 and tests were two-tailed unless otherwise noted. One-tailed tests were used for the predictions of greater TFR, TFAS, and shorter latency for cricket stimuli than for control stimuli. They are justified because Burghardt (1973) and Von Achen and Rakestraw (1984) made similar findings for *E. fasciatus*, and because *E. laticeps* repeatedly tongue-flick while foraging and often eat cryptic prey (Cooper and Vitt, 1986a) that might best be located by chemical cues. Numbers of trials in which biting occurred and other variables for which variances were heterogeneous were compared among conditions by nonparametric methods (Siegel, 1956) using multiple comparison tests described in Hollander and Wolfe (1973).

Data from a study of garter snake (*Thamnophis sirtalis*) responses to components of earthworm extracts (Burghardt et al., 1988) also were used to compare measures of feeding responses. In that study, seven snakes were tested for responses to seven stimuli presented on swabs in a counterbalanced order. Each stimulus was presented for a maximum of 30 sec. Number of tongue-flicks directed toward the swab, total tongue-flicks, and latency to attack were recorded. The stimuli were water, raw lyophilized earthworm extract (EW), and five reconstituted chemical fractions of earthworm extracts (B7, C7, D7, E7, and F7); the chemical nature of these is not relevant here. Data were analyzed as above for TFR, latency, PTFR, number of attacks, and TFAS.

RESULTS

Skinks. Lizards tongue-flicked and/or bit swabs in all conditions (Table 1) but had higher TFR and TFAS and shorter latency when responding to cricket chemicals than to control stimuli. ANOVA was not used to test differences in TFR among conditions because (1) Hartley's test showed significant heterogeneity of variance ($F_{max} = 6.35$; $df = 3, 11$; $P < 0.05$); and (2) although a square-root transformation somewhat reduced F_{max}, it remained significant and increased in magnitude when third roots were taken. Logarithmic transformation might have worked, but it was not performed. A Friedman two-way analysis of variance showed a significant stimulus effect (chi square = 8.67; $df = 2$; $P < 0.02$). Multiple comparisons revealed that the lizards emitted significantly more tongue-flicks in response to cricket chemicals than to cologne ($P = 0.0027$, one-tailed) or deionized water ($P = 0.037$ one-tailed). TFRs in response to water and cologne were statistically indistinguishable ($P > 0.10$, one-tailed).

TABLE 1. TONGUE-FLICKS IN 60 SEC (TF) AND LATENCY TO ATTACK IN SECONDS FOR RESPONSES BY ADULT MALE *Eumeces laticeps* TO CRICKET CHEMICAL STIMULI (CR), COLOGNE (CO), AND DEIONIZED WATER (DW)

	Response variables					
	TF Stimuli			Latency Stimuli		
Lizard	CR	CO	DW	CR	CO	DW
1	25	2	20	60	60	60
2	15	4	5	60	60	60
3	6	14	2	14	60	60
4	17	4	1	52	60	60
5	18	3	8	60	60	60
6	27	24	6	60	60	60
7	16	2	20	21	60	60
8	8	3	2	32	60	60
9	16	37	5	56	60	60
10	11	52	2	32	60	4
11	30	4	5	51	60	60
12	21	7	6	36	49	60
Mean	17.5	13.0	6.8	44.5	59.1	55.3
SE	2.1	4.1	1.9	4.8	0.9	4.7

Latency to bite was least for cricket chemicals (Table 1) but was not tested for significance parametrically because the variances were heterogeneous (F_{max} = 27.33; df = 3, 11; $P < 0.01$) and the heterogeneity increased after square-root transformation (F_{max} = 41.81; df = 3, 11; $P < 0.01$). A Friedman test revealed a significant stimulus effect (chi square = 11.60; df = 2; $P < 0.01$). After ties were eliminated, latency to bite swabs bearing cricket chemicals was shorter than for those bearing cologne in eight of eight trials and shorter than for deionized water in seven of eight trials. The difference was significant in multiple comparison tests for cologne (P = 0.028, one-tailed) but not water (P = 0.092). Latencies were similar in response to deionized water and cologne, there being 10 ties and one latency shorter for each stimulus ($P > 0.10$, one-tailed).

Eight skinks bit swabs in 10 trials. Eight bit in response to cricket chemicals. One skink bit in tests with each of the other stimuli; in these cases, the same lizard also bit during its trial with cricket chemicals. The attack on a swab bearing only water preceded a trial with cricket stimuli; that in a cologne trial followed a test with cricket stimuli. Effects of prior stimuli have been reported in snakes (Burghardt, 1969; Burghardt et al., 1988). No lizard bit in all three trials. An analysis of biting frequency was performed using binomial tests of the data cast in the manner of McNemar's tests for individual treatment comparisons (Siegel, 1956). Skinks bit in a significantly higher number of trials when the swabs bore cricket chemicals rather than either water alone or cologne (binomial P = 0.016, two-tailed). All four lizards that did not bite in any condition had higher tongue-flick rates when responding to crickets than to the other stimuli.

The TFAS showed the most pronounced responses by lizards to cricket stimuli for 11 individuals (Table 2). The remaining individual gave strong

TABLE 2. RESULTS OF THREE METHODS FOR ESTIMATING RELATIVE RESPONSE STRENGTHS FOR *Eumeces laticeps*[a]

	Response variables								
	TFAS			TFAS(R)			PTFR		
	CR	CO	DW	CR	CO	DW	CR	CO	DW
Mean	57.2	17.7	15.7	39.2	15.1	15.7	24.9	13.1	9.2
SE	8.5	6.2	8.6	5.6	4.9	8.6	2.7	4.7	2.6
Range	15–98	2–63	1–108	15–80	2–52	1–108	15–45.7	2–52	1–30

[a] Stimuli are CR (cricket chemical stimuli), CO (cologne), and DW (deionized water).

responses to all conditions. Variances were homogeneous, with Hartley's $F_{max} = 1.89$ ($P > 0.10$). The main treatment effect was highly significant ($F = 12.68$; $df = 2, 22$; $P < 0.001$). Comparisons of pairs of treatment means revealed that TFAS was significantly greater for the cricket chemicals than for deionized water or cologne ($P < 0.01$ each) but did not differ significantly between the two control conditions ($P > 0.10$).

The patterns of results and statistical significances for TFAS(R) were identical to those for TFAS (Tables 2 and 3). Variances were homogeneous ($F_{max} = 3.03$; $df = 3, 11$; $P > 0.05$), and the main stimulus effect was significant at $P < 0.001$ ($F = 10.37$; $df = 2, 22$). Cricket chemicals elicited significantly greater response than cologne or water ($P < 0.01$ each); mean TFAS(R)s to cologne and water were nearly identical ($P > 0.10$).

Results for PTFR (Tables 2 and 3) were similar in pattern to those for TFAS. Variances were homogeneous ($F_{max} = 3.21$; $df = 3, 11$; $P > 0.05$). The main effect was significant ($F = 5.04$; $df = 2, 22$; $P < 0.05$). PTFR was significantly higher in response to cricket chemicals than to cologne or deionized water ($P < 0.05$ each). There was no detectable difference in PTFR between water and cologne ($P > 0.10$).

The assumption of uniform tongue-flicking rates throughout the test interval was rejected for water ($t = 4.40$, $df = 20$, $P < 0.001$), cologne ($t = 3.97$, $df = 17$, $P < 0.001$), and male cloacal stimuli ($t = 2.42$, $df = 20$, $P < 0.05$), but not for female cloacal stimuli ($t = 0.65$, $df = 20$, $P > 0.10$). Mean tongue-flick rate in the initial 20 sec in response to commonly used control stimuli, i.e., water and cologne, is consistently higher than during the final 40 sec (Table 4). A similar but less pronounced trend is present for male cloacal stimuli. The high tongue-flicking rate in response to female cloacal stimuli was sustained for the entire 60 sec.

TABLE 3 COMPARATIVE PROBABILITIES AMONG RESPONSE VARIABLES OF NO SIGNIFICANT DIFFERENCES BETWEEN CONDITIONS FOR *Eumeces laticeps* CHEMICAL DISCRIMINATION DATA

One-tailed alpha for	Response variables					
	TFR	Latency	bites	PTFR	TFAS	TFAS(R)
Main effect	<0.010	<0.005	<0.005	<0.025	<0.001	<0.001
Cricket vs. cologne	0.038	0.092	0.078	<0.025	<0.005	<0.005
Cricket vs. water	0.003	0.028	0.078	<0.025	<0.005	<0.005
Cologne vs. water	>0.100	>0.100	>0.100	>0.100	>0.100	>0.100
Type of analysis	NP	NP	NP	P	P	P

TABLE 4. UNIFORMITY OF TONGUE-FLICK RATES DURING INITIAL 20 AND FINAL 40 SECONDS TO DEIONIZED WATER, COLOGNE, AND CONSPECIFIC CLOACAL STIMULI BY MALE *Eumeces laticeps*

Number of tongue-flicks	Stimuli			
	Water	Cologne	Male stimuli	Female stimuli
Mean initial 20 sec	1.90	4.39	6.24	11.19
Mean final 40 sec/2	0.52	0.36	3.19	9.88
Initial/final	3.65	12.19	1.96	1.13

Garter Snakes. Variances were homogeneous. Results were similar to those for the lizards in that TFAS allowed a better resolution of differences in response among conditions (Table 5). TFR did not differ significantly among stimuli, presumably because biting precluded further tongue-flicking to the most effective stimuli. The only significant differences in number of attacks were between

TABLE 5. RESULTS AND STATISTICAL COMPARISONS AMONG RESPONSE VARIABLES FOR SEVEN *Thamnophis sirtalis* TESTED ONCE EACH ON SEVEN DIFFERENT STIMULI[a]

S[b]	Response variables							
	TFR		PTFR		TFAS		Mean latency	Bites (*N*)
	s[c]	t[c]	s	t	s	t		
EW	5.1	9.7	11.5	12.9	29.4	41.2	15.3	6
WA	6.4	12.9	6.4	14.7	6.4	12.9	30.0	0
B7	8.9	14.7	14.2	15.1	17.1	26.4	24.7	2
C7	6.4	19.4	6.4	19.3	6.4	19.4	30.0	0
D7	6.1	14.7	6.1	19.4	6.1	14.7	30.0	0
E7	7.4	9.7	12.8	21.0	19.8	28.6	22.3	4
F7	7.1	12.9	14.0	21.1	24.7	39.0	18.6	5
F	0.51	1.13	2.57	1.30	7.39	6.09	[d]	
P[e]	>0.10	>0.10	<0.04	>0.10	<0.0001	<0.0002	<0.02	

[a] Data from Burghardt et al. (1988, Table 2) and related experiments.
[b] S-stimuli: water (WA), raw lyophilized earthworm extract (EW), and several chemical fractions of EW (B7, C7, D7, E7, and F7).
[c] s-tongue-flicks to swab; t-total tongue-flicks.
[d] Friedman two-way ANOVA was used due to several zero variances.
[e] *P* values are two-tailed; $df = 6, 36$ for *F* tests (used for tests of all variables except latency and number of bites).

EW and the three stimuli that elicited no attacks, water, C7, and D7 (one-tailed binomial $P = 0.016$) and between E7 and the stimuli eliciting no attacks ($P = 0.031$). No test of the main effect of biting was conducted because of the low expected cell frequencies. Latency could not be analyzed parametrically because of zero variance for the three extracts that failed to elicit biting. However, a Friedman two-way ANOVA revealed significant variation in mean latency among stimuli ($\chi^2 = 16.59$, $df = 6$, $P < 0.01$). For total tongue-flicks and tongue-flicks directed only to the swab, F values are largest and alpha values lowest for TFAS. If the same data are analyzed by Friedman two-way ANOVA, the rank order of relative magnitudes of alpha values among stimuli is preserved for both total tongue-flicks and tongue-flicks to swabs. P values are slightly larger in the nonparametric analysis. Higher F values were obtained for TFAS and PTFR, but not TFR when only tongue-flicks were directed toward the swab, rather than all tongue-flicks, including those directed away from the swab, were used.

DISCUSSION

Comparisons of Response Measures

Skinks. TFR, latency to bite, number of lizards biting, PTFR, TFAS and TFAS(R) were concordant in the skink study in detection of a significant difference among stimulus conditions and between responses to cricket chemicals and deionized water and of no significant difference between responses to water and cologne. However, using the same data set, the ability of these measures to resolve other differences in responses among conditions varied; similarly, suitability of the data for parametric analysis differed among the measures (Table 3). The variables allowing maximal detection of differences in responses among conditions were TFAS, TFAS(R), and PTFR, in descending order. Although patterns of results were similar for all variables, number of lizards biting could not be adequately analyzed without a larger sample size, and the multiple comparisons for latency and TFR were significant only at higher alpha levels than for TFAS, TFAS(R), and PTFR.

Response to cricket chemicals differed significantly from that to cologne for all variables except latency to bite, for which the difference was not significant, in part because of the failure of several individuals to bite. Latency alone should be relatively uninformative in revealing treatment effects for prey chemicals failing to elicit biting in a high proportion of trials. Its effectiveness is reduced whenever some individuals also bite control swabs. A further consideration is that variances will be zero in conditions not eliciting biting and will be low in conditions eliciting infrequent biting. The usefulness of number of lizards biting is similarly limited. Furthermore, it is a nominal variable, whereas

the others considered are at least ordinal. Relatively large sample sizes are required to meet the assumptions of the Cochran Q test if more than two conditions are tested. At best, important information available from tongue-flicking is discarded when latency is used; further quantitative information regarding latency is discarded in using number of lizards biting.

Biting is important in two ways, as an end point for trials and as a presumed feeding attempt. Once a lizard has bitten, the trial ends because tongue-flicking before and after biting may not be commensurate, because it is difficult to determine or interpret the duration and number of bites, and, most importantly, because biting is a natural termination of prey capture. Nevertheless, some individuals bite swabs not bearing prey chemicals. Fortunately, such behavior is relatively rare. In studies of several other snake and lizard species, either no bites were directed to control stimuli or they were rare and usually occurred in trials immediately following testing with attack-eliciting prey extracts (Burghardt, 1973; Burghardt et al., 1988; Cooper, unpublished). Prey-directed strikes usually are unambiguous and motivated by hunger (Burghardt, 1970), whereas tongue-flicking is affected by various contextual variables, including novelty (De Fazio et al., 1977; Gove and Burghardt, 1983; Burghardt et al., 1986; Wellborn et al., 1982).

Results for TFR and PTFR were similar. Minor differences in alpha levels may have been due in part to differences in the Friedman test and ANOVA as well as to inherent differences between variables. TFR and PTFR suffer from loss of all biting information. Raw numbers of tongue-flicks inadequately represent trials in which biting occurs, the inadequacy increasing as latency to bite decreases. The use of PTFR addresses this difficulty by projecting each animal's TFR to the time of biting through the entire 60-sec interval. This projection should improve as latency increases.

Equal TFRs for the intervals before and after biting are assumed in PTFR. This assumption of uniform tongue-flicking rate is invalid for the control stimuli water and cologne and for conspecific male cloacal stimuli, but it is valid for female cloacal stimuli. Thus, uniformity cannot be assumed and should be determined empirically for each stimulus used. Violation of the uniformity assumption for water and cologne would have minimal effects because these stimuli rarely induce biting in a variety of species (Cooper, 1989a,b; unpublished; Cooper and Vitt, 1989), but it would have a greater effect for prey chemicals. PTFR becomes increasingly unrealistic as the departure from uniformity increases and as the proportion of lizards biting increases. PTFR could be improved by recording the temporal distribution of tongue-flicks and applying correction factors for each stimulus found to have a significantly nonuniform rate.

Results for TFAS and TFAS(R) were nearly identical, both separating con-

ditions at lower alpha levels than did PTFR. As expected, the *F* value for TFAS was slightly greater than for TFAS(R) because a constant maximum number of tongue-flicks was added to the TFAS for every individual that bit, whereas a variable and usually lower number was added to arrive at TFAS(R). TFAS, although appropriate for independent group designs, somewhat inflates group differences for studies involving repeated measures of performance by the same individual. Because a major advantage of the randomized blocks design is matching of responses across stimuli within individuals, this advantage is lost by assigning a value for one individual to other individuals. Therefore, TFAS(R) may be preferable to TFAS for use with randomized-block designs. Some individuals that attack but tongue-flick little may have a lower TFAS(R) than others that never attack, but this cannot be so for TFAS. In practice, there may be little difference between the two. However, for the nonparametric Friedman two-way ANOVA, which compares ranks of each individual across conditions, TFAS(R) would be best.

Garter Snakes. Results differed from those in the skink study in that TFR did not differ among stimuli and PTFR produced only a marginally significant main stimulus effect. Thus, whereas similar results were obtained using TFR or PTFR for the skinks, results from these variables are discrepant for the garter snakes. This difference is attributable to the shorter latency among those individuals that attacked for the snakes, resulting in low TFR and a greater increment in PTFR.

TFAS gave a much better overall picture of differences in responsiveness to the various stimuli than the other measures. Because such a high percentage of garter snakes attacked prey stimuli at fairly short latencies, the number of individuals attacking swabs is a better response index than TFR alone. With sufficient sample size to allow assessment of main effects nonparametrically, number of attacks might be preferable to TFR and PTFR for this species. Latency appears to be the best single-component measure for these data because it differs markedly among stimuli, is amenable to analysis of the main effect, and is suitable for multiple comparisons. Latency is a priori a poorer measure than TFAS because it fails to account for variation in tongue-flicking rate. Practically, it is inferior because it is more likely to require nonparametric analysis and because it yields larger alpha values (as seen for both data sets).

Comparison of differences among conditions has been based on data for all individuals, including latencies for those that do not attack. We may also wish to ascertain whether the latency to attack varies among stimuli given that attack has occurred. For the garter snake data, this conditional latency does not appear to differ among stimuli. Mean latencies were 12.9 for earthworm extract, 11.4 for extract B7, 16.6 for extract E7, and 14.0 for extract F7. No analysis was conducted because the data were a mixture of independent and correlated

observations. Nevertheless, it is clear that (1) results differ greatly from those including all latencies and (2) all latencies should be included when assessing whether response strengths vary among stimuli.

That the *F* value for TFAS based on tongue-flicks directed to the swab was greater than that for total tongue-flicks suggests that the former better reflects response to chemicals. We have observed in many studies that snakes and lizards sometimes tongue-flick while moving about the cage, without apparent reference to stimuli on the swabs. Some individuals give the impression of searching for the prey object by tongue-flicking away from the swab; others may be escaping or merely moving away. Because it is difficult to distinguish the motivation for tongue-flicks away from the swab and because tongue-flicks away from the swab may also differ among stimuli, tongue-flicks directed to the swab give the best indication of response to prey stimuli.

Characteristics of Good Index of Response Strength

TFAS has the advantages of giving bites heavier weight than any number of tongue-flicks. PTFR, if modified to account for the temporal distribution of tongue-flicks, might give a somewhat less arbitrary weight in terms of tongue-flicks for the interval following biting. Because, however, it would not allow additional weight for biting, the adjusted PTFR would be inferior to TFAS and TFAS(R). When the tongue-flick rate is highest in the early part of the trial, PTFR effectively weights early biting heavily. In this case TFAS is preferable because the degree of weighting is unknown for PTFR. A measure of response strength combining the features of a modified PTFR with some nonarbitrary weight for biting in terms of tongue-flick units could be a further improvement.

If we ask how many tongue-flicks a bite is worth or what fraction of a bite a given tongue-flick and latency combination is worth, it becomes clear that the units must be arbitrary. Yet it is possible to specify properties of a good measure of response strength to prey chemicals combining the effects of tongue-flicking, latency to bite, and biting. They include: (1) monotonic increase with increases in number of tongue-flicks, (2) monotonic increase as latency decreases, and (3) greater magnitude for trials involving biting than for any number of tongue-flicks alone.

TFAS and TFAS(R) have these properties with the single exception noted above; PTFR has property 1, has property 2 unless biting occurs with zero latency, but does not have property 3. For a bite at zero latency, no tongue-flicking rate has been established for projection through 60 sec. However, bites at zero latency occur very rarely, and only in experiments in which the test interval is considered to begin with the first tongue-flick or bite rather than immediately upon introduction of the stimulus. A related problem with PTFR is that for short latencies, the projection is less likely to be realistic. Because

of these shortcomings and the additional data and computation required for a projection reflecting the true temporal distribution of tongue-flicking, PTFR is a less satisfactory measure than TFAS or TFAS(R). PTFR could be modified to handle zero latency by adding some base unit, but this would impose an additional arbitrary element. Furthermore, unless a base unit were to be added for biting as in TFAS, it would be possible for one individual to have a higher PTFR in a condition in which it did not bite than for one in which it did. Halpern and Frumin (1979) developed an index adding a latency component: response strength = PTFR + 2(60 − latency to attack).

This measure is similar to TFAS except that PTFR is substituted for the base unit and the latency component is multiplied by two. We prefer TFAS and its derivatives to this index because of the problems with PTFR discussed above.

Characteristics of TFAS and its Relationships to Tongue-Flicks, Biting, and Latency

For individuals that do not bite, TFAS is simply the number of tongue-flicks emitted; for individuals that do bite, it is the base unit (maximum number of tongue-flicks in the study) plus (60 − latency to bite). The portion of the TFAS function in the absence of biting coincides with the 60 sec latency in Figure 1. A wide TFAS range is possible when no biting occurs. When a bite does occur, there is a unique TFAS associated with each latency. A negligible difficulty with TFAS is that a squamate biting at exactly 60 sec could receive a score identical to that in the trial with the most tongue-flicks.

Relationships between TFAS, TFR, and latency are strongly affected by the frequency and timing of biting. If no individuals bite, TFAS and TFR are identical. As the proportion of individuals biting in response to prey chemicals increases and the mean latency decreases, TFR gives an increasingly poorer indication of responsiveness to prey chemicals and diverges from TFAS. If all individuals bite after a few tongue-flicks, it is possible to have lower TFR to prey chemicals than to control stimuli.

If all animals bite swabs bearing prey chemicals, latency and TFAS are perfectly correlated; with decreasing proportions of individuals biting in response to prey chemicals, the correlation decreases, reaching zero if no individuals bite.

In the foregoing, it is assumed that biting occurs solely in response to prey chemicals. In studies involving 11 species of lizards representing eight families and two species of colubrid snakes (Cooper, unpublished), biting of control swabs was rare. Two *Eumeces laticeps* bit a control swab (Cooper and Vitt, 1989); two other individuals were discarded from the study because they bit immediately without any preliminary chemical testing. A single *Tupinambis rufescens* could not be used because it attacked any small object introduced into

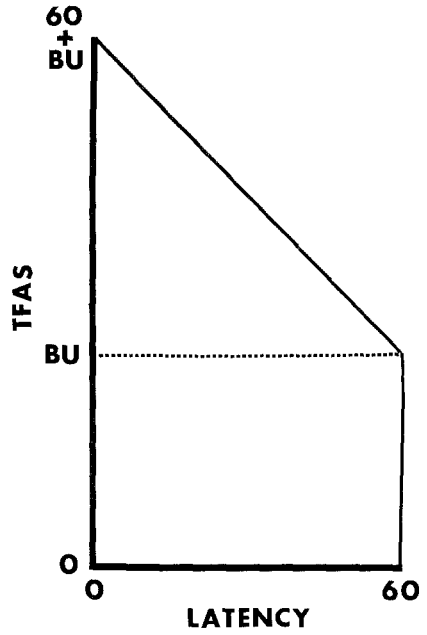

FIG. 1. The tongue flick/attack score (TFAS) consists of two components: the number of tongue-flicks for individuals that do not bite, and a base unit + (maximum trial length − latency) for those that bite. The base unit (BU), which is the maximum number of tongue-flicks emitted by any lizard in the entire sample in response to any stimulus condition, is constant for a given study but varies among studies. Trial length shown is 60 sec.

its cage without prior chemosensory investigation. Infrequent biting of swabs bearing control substances has not created any difficulty in detection of prey chemical discrimination to date but could do so in a species that bit control swabs frequently. This might occur in highly aggressive species that bite defensively (e.g., Herzog and Burghardt, 1986).

Published analyses of TFAS data have used both parametric and nonparametric statistical methods. Because TFAS and TFAS(R) are unlikely to be interval scales, nonparametric analyses are safest. In a stringent nonparametric approach, medians would be presented rather than means and variance-based parameters would be unavailable. One way to avoid this difficulty would be to construct an interval scale of response using psychological scaling techniques involving normalized TFAS, but this would demand much larger sample sizes and would involve considerable additional calculation. These disadvantages seem to outweigh the potential gains.

Parametric ANOVA is robust enough with the nearly interval data on TFAS

to warrant its use if variances are homogeneous or can be rendered homogeneous by transformation. In the numerous instances in which we have analyzed the same data by parametric and nonparametric methods, the results have been equivalent. When nonparametric tests are used for experiments involving three or more groups, a need for significance tests for individual pairs of treatment or condition means arises if the main effect is significant. However, most statistics books used by biologists do not describe appropriate multiple comparison tests. However, such tests are available (Hollander and Wolfe, 1973) and should be used with Kruskal-Wallis and Friedman ANOVAs.

Adjustment of Scores for Biters to Base Unit Differences

A different approach to improvement of TFAS is standardization of the relationship between the base unit and the latency component. When biting occurs, the contribution of latency to TFAS is constant between all studies having the same trial length, but the base unit may vary among studies. If the base unit is large relative to the latency contribution, biting will affect TFAS less than if the base unit is relatively small. However, because the base unit depends on the extreme response of one individual and on factors such as food deprivation and temperature, it may be expected to be quite variable.

TFAS may be adjusted to maintain a constant ratio between the maximum possible contribution of latency and the base unit. The new base unit-adjusted TFAS, BTFAS, is given by

$$BTFAS = \text{base unit} + c(TL - \text{latency})$$

where c is a latency constant and TL is trial length in seconds. To give equal weight to the base unit and immediate biting (so that maximum BTFAS = 2 × base unit),

$$c = \frac{\text{base unit}}{TL}$$

For TFAS, the proportion of the total variance attributable to biting and its latency increases with the ratio of the base unit to (TL − latency) because multiplication of data by a constant increases the variance by the square of the constant. In similar conditions the mean increases by the constant of multiplication. Results based on BTFAS are more comparable between studies than those based on TFAS because the proportion of total BTFAS due to biting at a given latency is identical in all studies. Because the latency coefficient c is applied only to scores involving biting, effects on mean and variance increase with the proportion of individuals biting and the deviation of the base unit to (TL − latency) ratio from unity.

BTFAS and TFAS were calculated (with TL = 60) and statistical tests

were conducted to obtain preliminary information regarding effects on outcomes of ANOVA for the *E. laticeps* data and three smaller hypothetical data sets. The data sets varied in values of the latency coefficient, proportion of individuals biting in response to prey chemicals, and latencies. Magnitudes of F values obtained for each data set were compared by computing the ratio of F(BTFAS) to F(TFAS). Results for each data set are reported for both randomized blocks and independent groups analyses (Table 6).

These examples show that F values may increase or decrease when c is not equal to 1.0. Relatively large changes occurred for samples in which c deviated substantially from 1.0. For comparisons based on the same data set, the relative values of F for BTFAS and TFAS differed only slightly for repeated measures and independent groups analyses when c was close to 1.0, but to a larger extent for the c values far above or below 1.0. This shows that F values are sensitive to the proportion of the total response measure assigned for effects of biting and latency and that the effect is complex, presumably varying with c, experimental design, and proportion of animals biting.

BTFAS for the *E. laticeps* data set gave results very close to those for TFAS because the base unit (52) was close to 60, giving a c value of 0.8667. BTFAS means $\pm$ SE and ranges were 55.5 $\pm$ 8.0 and 15–91.9 for cricket stimuli, 17.5 $\pm$ 6.2 and 2–61.5 for cologne, and 15.0 $\pm$ 8.0 and 1–100.5 for deionized water. Variances were homogeneous ($F_{max} = 1.69$; $df = 3, 11$; $P > 0.10$). The pattern and levels of statistical significance for BTFAS were identical to those reported for TFAS. However, the main effect was slightly larger for BTFAS ($F = 13.06$; $df = 2, 22$; $P < 0.001$, F ratio = F(BTFAS)/F(TFAS) = 1.03). The individual comparisons between cricket stimuli and control stimuli also exceeded critical values by larger margins than in similar comparisons for TFAS.

TABLE 6. RELATIVE MAGNITUDES OF F VALUES FOR TFAS AND BTFAS VARIED EVEN WITH IDENTICAL DATA SETS[a]

Sample size[b]	Proportion biters	c[c]	F(BTFAS)/F(TFAS) Randomized blocks	F(BTFAS)/F(TFAS) Independent groups
4	0.50	0.5000	1.31	1.36
12	0.75	0.8667	1.03	1.04
4	0.50	1.1667	1.05	1.04
4	0.50	2.0000	0.86	0.76

[a] Each data set was analyzed using independent groups and randomized blocks designs.
[b] Sample size is 12 for the *E. laticeps* data, 4 for hypothetical data sets.
[c] c is the latency coefficient.

Among studies done in a comparative survey of prey chemical discrimination (Cooper, unpublished) in which at least one individual bit, base units for lizards in 60-sec tests were 15, 23, 30, 31, 48, 52, and 59; for a single snake study it was 47. For several of these studies c would be considerably below 1.0 (as low as 0.25) because the base unit is considerably smaller than 60. The short trial period limits the size of the base unit, but a potential for base units considerably larger than 1.0 is indicated by the 116 tongue-flicks emitted in 60 sec by a male *E. laticeps* responding to conspecific female cloacal stimuli (Cooper and Vitt, 1986b).

Recommendations

Comparisons of response variables show that Burghardt's (1967, 1970) widely used TFAS or a slightly modified version of it is the best index of response by squamates to prey chemicals on swabs. Continued use of TFAS or TFAS(R) is acceptable, but for comparison of results between studies of multiple species or populations, BTFAS may be preferable. A drawback of BTFAS is that the decision to give an immediate bite twice the weight of the base unit is arbitrary.

It is also suggested that the base unit be changed so that a bite at exactly 60 sec latency would receive a greater TFAS than the greatest number of tongue-flicks in the absence of biting. All versions of TFAS could be slightly improved by adding a small constant to the current base unit. If no individuals bite at exactly 60 sec, which is the case for the data set analyzed here and for many others, the current base unit is adequate. Because intervals of less than 1 sec are not usually recorded, addition of 0.5 to the base unit for TFAS or of $0.5c$ for BTFAS would suffice. Alternatively, an arbitrary deduction of 0.1 sec from latency of a bite at 60 sec would accomplish the same thing. One of these minor changes is suggested only to satisfy property 3 in a rare case. It is highly unlikely that the suggested change in base unit will have any appreciable effect. We plan to use TFAS, TFAS(R), BTFAS, or BTFAS(R) without modification in future studies unless one or more bites have 60-sec latencies, in which case the base unit will be increased as stated.

Solely for detection of prey chemical discrimination, separate analysis of latency to bite and number of bites does not appear to provide information in addition to that obtained from TFAS. However, to reach a thorough understanding of the behavioral responses to chemical stimuli, attention must be paid to separate information on tongue-flicking rate, latency, and biting and their interrelationships (Cooper and Vitt, 1989). Information on all these variables should be included in papers on squamate prey chemical discrimination.

Experimental procedures used in recording tongue-flicks and initiating trials may affect the magnitudes of all response measures considered here. For exam-

ple, trials may be initiated immediately upon presentation of the stimulus (Burghardt, 1975) or upon emission of the first tongue-flick (Cooper and Vitt, 1989). The rationale for the latter procedure is that defensive immobility in the presence of the experimenter may initially prevent tongue-flicking. Use of a behavioral starting point eliminates this problem, but prevents zero scores and is not feasible for species that frequently fail to tongue-flick swabs (e.g., *Anolis carolinensis*, *Sceloporus malachiticus*, *Calotes mystaceus*, Cooper, 1989a).

A second procedural consideration is whether to touch the reptile's snout with the swab if it does not tongue-flick after some predetermined interval. Touching may induce tongue-flicking by individuals that would otherwise be unresponsive, but in some species it induces defensive bites (Cooper, unpublished observations on *E. laticeps* and *Masticophis flagellum*).

Another source of variation among studies is the categories of tongue-flicks recorded, i.e., all tongue-flicks or only those directed to the swab (e.g., Burghardt, 1975). Among lizards some species direct most tongue-flicks to the swab (e.g., *E. laticeps*, Cooper, unpublished); some may direct a few tongue-flicks to the swab and then move away while continuing to tongue-flick, sometimes at high rates (*Ophisaurus ventralis*, *Cnemidophorus sexlineatus*, *C. tigris*, Cooper, unpublished); others may tongue-flick swabs repeatedly, move away while tongue-flicking, and return (*Heloderma suspectum*, *Tupinambis rufescens*, Cooper, 1989b). Similar variation exists in snakes. In garter snakes (*Thamnophis sirtalis*) most tongue-flicks for effective stimuli are directed to swabs. In *Elaphe guttata* a higher proportion of tongue-flicks is directed elsewhere. Given such procedural differences and their potential effects on response variables, caution must be used in making comparisons between studies, populations, or species.

Other Measures not Analyzed Here

Other response measures may be constructed, including multiplicative relationships between tongue-flicks and latency. One such would be the number of tongue-flicks if no attack occurred or the maximum number of tongue-flicks for the group (or each individual) multiplied by [trial length/(trial length − latency)] if attack occurred. Such measures would be infinitely large for attack at zero latency. A potentially important factor in such indices might be the number of attacks divided by mean latency (Burghardt, 1975). Whether the relationship between latency and the base unit should be additive, as in TFAS, or multiplicative is uncertain. However, TFAS and its modifications are likely to yield smaller differences among stimuli. Means of additive or multiplicative measures could be further adjusted for the proportions of individuals biting prey and control stimuli. Such adjustment could be useful in comparative studies,

but effects of biting would be entered for both individual trials and entire conditions.

TFAS and its modifications are composite variables derived rationally from ethologically reasonable premises about the importance of tongue-flicking and attack as indicators of feeding response strength. Another approach would be to use multivariate methods to select weights for each response variable empirically to yield the maximum possible statistical discrimination among conditions or treatments, as in MANOVA. We prefer the rational approach because statistical compositing procedures may capitalize on features unique to each data set. Use of different relative weights in each study would make comparisons among studies unnecessarily difficult.

We have not considered cases in which each individual is tested more than once with each stimulus. For such data, Halpern and Frumin (1979) use a preference score, which is the difference in mean response strength for prey extracts and water expressed as a proportion of mean response strength to water. This method was used in an attempt to eliminate effects of differences in response rates among snakes and to control for indiscriminate responses, i.e., responses not attributable to prey extract. Such a complex approach is not necessary to detect prey chemical discrimination in experiments using randomized blocks designs because each individual serves as its own control. Use of preference score requires the assumption that equal ratios (difference in response scores/water response score) indicate equal response to prey chemicals regardless of the overall response rates. This is unlikely to be true in the general case because at high response scores for water, it would be impossible for subjects to tongue-flick and/or bite rapidly enough to attain as high a ratio as would be possible with lower response scores to water.

In addition to the measures of response strength used in experiments in which chemical stimuli are presented on swabs, a tongue-flick interest score (Reformato et al., 1983; Halpern et al., 1984) incorporating number of tongue-flicks and relative amounts of time spent at two sample trays has been used for simultaneous discrimination experiments. This method appears to be reasonably efficient, but its merits relative to the procedures used in successive discrimination procedures with chemical stimuli on swabs are unknown. Experimental results based on tongue-flick interest scores and tongue flick-attack scores are unlikely to be directly comparable. TFAS includes no component similar to time spent at a tray, which is reminiscent of the orientation time to a swab recorded as a measure of response strength in an early study by Loop and Scoville (1972). Because this measure does not account for either number of tongue-flicks or latency to attack, it has not been widely used. The tongue-flick interest score includes the influence of numbers of tongue-flicks, but does not account directly for attacks (which do occur in this procedure) or their latency. Although

a response measure including the effects of latency and attack might be developed for use in simultaneous discrimination tests, there is no guarantee that it would give results comparable to TFAS and no necessity that it should.

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ANALYSIS OF CHEMICALS FROM EARTHWORMS AND FISH THAT ELICIT PREY ATTACK BY INGESTIVELY NAIVE GARTER SNAKES (*Thamnophis*)

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Abstract—Materials previously shown to elicit increased tongue-flicking and prey attack in garter snakes (*Thamnophis sirtalis*) were isolated from both earthworms (*Lumbricus terrestris*) and fish (*Pimephales promelas*). Both high- and low-molecular-weight components from earthworms and fish stimulated attacks and increased tongue-flicking in previously unfed neonate garter snakes relative to distilled water controls. Earthworm collagen was also effective, but even concentrated fractions were less effective than raw extract. Conflicting reports on the effectiveness of collagen suggest that the salient chemical(s) is a smaller molecule tightly bound to collagen and resisting standard purification methods.

Key Words—Chemoreception, garter snakes, *Thamnophis sirtalis*, chemical cues, *Lumbricus terrestris*, earthworm, *Pimephales promelas*, fish, prey odors, glycopeptide.

INTRODUCTION

Snakes of the genus *Thamnophis*, as well as many others, use chemical cues to discriminate among prey items (Burghardt, 1970, 1980; Arnold, 1981). Surface chemicals can be removed from various prey and used to elicit feeding responses. The most common method involves presenting aqueous prey extracts

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to snakes on cotton swabs and recording the number of tongue-flicks and the latency of any prey attack (Cooper and Burghardt, 1990). Extensive comparative studies show that newborn garter snakes respond to a wide range of prey chemicals highly correlated with the normal prey preferences of particular species in captivity and in the field (e.g., Burghardt, 1969). The tongue–vomeronasal organ system is responsible for these discriminations (Burghardt, 1970; Halpern and Kubie, 1984).

In an earlier paper (Burghardt et al., 1988), we reviewed the current status of the chemistry of the relevant prey cues and presented our results on a comparison of the chemicals extracted from two prey species, a fish (*Pimephales promelas*) and an earthworm (*Lumbricus terrestris*). Halpern et al. (1986) reviewed the chemistry of earthworm recognition in adult common garter snakes (*Thamnophis sirtalis*) based on their extensive work. They report that purified earthworm cuticle collagen is a potent source of chemoattractant for garter snakes and identify the main effective component as a collagen-like substance (Kirschenbaum et al., 1986). However, using highly purified collagen, this result could not be replicated in adult common garter snakes with our bioassay (Goss, 1986). Two factors may contribute to the discrepancy: the two methods of extraction now used [aqueous extract (Sheffield et al., 1968) and the recently reported chloroform–methanol method (Burghardt et al., 1988)] and the different bioassays employed by the two laboratories to define activity.

The bioassay issue has been discussed in Burghardt et al. (1988). In brief, our method involves a successive discrimination task with open-mouth prey attack being the definitive response in contrast to a simultaneous discrimination test based primarily on tongue-flick rate (Halpern et al., 1986). Any differences in chemical results between the two laboratories may be due to the bioassay employed and may account for our discovery of an active low-molecular-weight fraction in the aqueous extract that has not apparently been observed by the Halpern group.

The chloroform–methanol extraction method (Burghardt et al., 1988) provides especially high yields of active low-molecular-weight materials. These clearly differ in molecular weight from the principal component of the aqueous extract, but are equally or more effective than the low-molecular-weight component(s) of the aqueous extract in bioassay results. An amino acid and carbohydrate analysis showed only partial similarity of low- and high-molecular-weight components to the reported constitution of collagen. One explanation is that the three substances are different and that garter snakes respond to all three.

To date, no single investigation has demonstrated that garter snakes from the same population respond to low- and high-molecular-weight fractions and collagen derived from earthworms. We demonstrate this here and must now question the simple explanation noted above. An alternative explanation would suggest that each fraction contains a component(s) that has the requisite struc-

ture for recognition by garter snakes. Specific structural details have not yet been determined; however, occurrence of activity only in fractions reflecting a peptide and carbohydrate composition clearly suggests a glycopeptide nature.

The ability of garter snakes to discriminate among extracts from different prey implies that more than a single chemical cue is involved. Even within the same species, initial and experience-altered preferences show that individual snakes discriminate chemical cues from different prey (e.g., Burghardt, 1970). In the present study, we compare the effective chemicals from two phylogenetically diverse prey: a fish, the fathead minnow, and the well-studied *Lumbricus* earthworm. We have previously shown that adult snakes respond to both high- and low-molecular-weight fractions from both prey, but not to earthworm collagen (Goss, 1986; Burghardt et al., 1988). Ingestively naive newborn snakes have not previously been tested. All the results presented here are based on neonates.

METHODS AND MATERIALS

Animals. Surface extracts were prepared from freshly obtained adult earthworms (*Lumbricus terrestris*) and fathead minnows (*Pimephales promelas*).

Neonate eastern garter snakes (*Thamnophis s. sirtalis*), born in the laboratory to commercially obtained females from Michigan (Wayne County), were used in the bioassay. All animals were individually housed and tested in their home cages (styrene boxes measuring 18 × 13 × 5 cm). Each box contained a commercial cage liner, a water dish, and a shelter made from a small piece of folded cage liner. Snakes were tested at a temperature of 25 ± 2°C. The snakes had not previously been fed and were between 15 and 20 days old when tested.

Aqueous Prey Extractions Procedure. Preparation of aqueous prey extracts followed Burghardt (1969). We extracted intact prey animals for 2 min with a ratio of 3 g of prey to 10 ml of distilled H_2O at 60°C. The solution was decanted and centrifuged for 10 min at 2500 rpm. The aqueous extract was then lyophilized to preserve activity. The amount of material obtained from prey ranged from 2 to 3 g/liter. References to this aqueous extract obtained from the earthworm and fathead minnow will be referred to as AQ-EW and AQ-FM respectively.

Chloroform–Methanol Prey Extractions Procedure. In this procedure, 1000 g of prey was extracted for 4 min at 23°C with a solution containing 2000 ml of methanol and 1000 ml of chloroform. The solution was decanted and the prey extracted again with 1000 ml of chloroform for 4 min. The decanted chloroform solution was combined with the original organic solution, and the total organic extract was filtered with a coarse glass frit to remove particulate matter.

The filtered solution was poured into a 5-liter separatory funnel and vigorously shaken with 1000 ml of distilled H_2O. The resulting emulsion was allowed to stand at 4°C, during which time it separated into two layers. After two days, the biphasic solution was allowed to warm to room temperature. The water layer was removed and filtered with a medium glass frit. The water layer was lyophilized and the organic layer was evaporated to dryness at aspirator pressure using a rotary evaporator. About 3–5 g of material was obtained per kilogram of prey, and approximately 30% and 40% by mass of these materials were obtained from the earthworm and fathead minnow water layers, respectively. This extraction procedure is referred to as the CM extraction.

Chromatographic Separations. All chromatographic separations, unless otherwise stated, were conducted with low-pressure liquid chromatography. The system included an FMI pump (model RP-SV), Laboratory Data Control refractive index detector (model 1107H), and a Scientific Manufacturing Industries fraction collector (model 12050).

Separation of the high- and low-molecular-weight fractions of earthworms and fathead minnows was conducted using Bio-Rad P-10 gel, 50–100 mesh with 10% ethanol elutant according to Burghardt et al. (1988).

Preparations of Cuticle Collagen. Two procedures were used to obtain collagen samples from earthworms. In the first, based on Murray et al. (1982), 100 earthworms, 15–20 at a time, were frozen for 20 min using a Dry Ice–acetone bath. The frozen earthworms were placed in distilled H_2O and allowed to thaw overnight at 5°C. The cuticles were removed and washed several times with distilled water, minced, and ground in a mortar with 0.5 M sodium chloride solution. The resulting slurry was stirred in 100 ml 0.5 M sodium chloride solution for 48 hr at 5°C and then centrifuged to remove insoluble materials. The supernate was submitted to ammonium sulfate precipitation to isolate the collagen as a pellet after centrifugation. This was reprecipitated twice from 0.5 M sodium chloride solution and finally reconstituted in distilled water for testing. This was a method used by Kirschenbaum et al. (1986) in obtaining their positive results.

A second collagen preparation, based on the procedure of Muir and Lee (1970), also was used to obtain test material. In this procedure, the cuticles were lyophilized prior to extraction with sodium chloride solution. The remainder of the preparation followed the procedure outlined above. Goss (1986) obtained negative results with this method.

Bioassay Procedures. The number of snakes tested in each bioassay was four or ten. All had responded to fish or earthworm aqueous extracts in a survey carried out on a previous day using the same testing procedure. Each snake was tested only once with each stimulus in a given bioassay session, with order in sequence equalized as completely as possible.

All extracts were reconstituted in distilled H_2O to a concentration repre-

senting, as near as possible, that found in the original extracts, less estimated losses on columns, etc. Each bioassay session included four or five stimuli; it always included a distilled water control and an AQ extract of the prey being tested. All tests were conducted blind.

In the bioassay, a 15-cm wooden cotton-tipped swab was dipped in the extract and introduced into the snake's home cage about 2 cm from its snout. It remained in that position as closely as possible (by following snake movements) for 30 sec or until the snake gave an open-mouth attack. The number of tongue-flicks directed towards the swab, total tongue-flicks, and the latency of any attacks were recorded. If the snake did not tongue-flick in 10–15 sec after being presented with a swab, the end of the swab was brushed lightly on the snake's back to induce it to emit at least one tongue-flick. For any bioassay session the time between tests was kept to 10–12 min. This basic methodology has been used in many studies (see Cooper and Burghardt, 1990).

Statistical analysis was accomplished by comparing responses to test extracts with distilled water controls and to the AQ extract. The water control represented no activity, whereas the AQ extract represented strong activity. The measures used for the statistical analyses were the number of attacks, attack latencies, and the number of tongue-flicks each snake exhibited toward each stimulus. These were combined into a tongue-flick/attack score (TFAS) (Burghardt, 1969) in which any attack received a higher score than any number of tongue-flicks. In brief, TFAS = base unit + (30 − attack latency) where the base unit was the maximum number of tongue flicks given in any nonattack trial by any snake in a given experiment.

A repeated measures analysis of variance (stimulus × subject × position in sequence) was performed on the data for all the extracts tested each bioassay session. All variances in an experiment were homogeneous according to Cochran's C test. However, a nonparametric Friedman's two-way analysis of variance also was performed. If both overall ANOVAs were significant, planned comparisons of prey extracts with water were made. A nonparametric Wilcoxon signed rank matched pairs test confirmed all paired comparisons conclusions. One-tailed P values are presented for these comparisons since in all cases only an increase in responsivity compared to water was being evaluated. All statistical calculations were performed using the Statgraphics package (Version 2.6).

RESULTS

Earthworm Extracts. Extraction of prey with organic solvents followed by extraction of the organic layer with H_2O provided active material easier to chromatograph than material obtained by direct H_2O extraction. The organic layer from the chloroform–methanol prey extract was not active, whereas the water

layer was very active. Compounds obtained from the H_2O layer were separated on the Bio-Gel P-10 column into two fractions: a multicomponent high-molecular-weight fraction eluting at the void volume of the column and a low-molecular-weight fraction eluting approximately at the volume of the column.

Two low-molecular-weight fractions were obtained after we noticed a change in the detector response as a function of the reference cell solution composition. The tested fractions represent a maximization of this difference as the reference is changed from the elution mixture (10% ethanol) to pure H_2O.

Ten snakes were tested on five stimuli with position in test sequence equated. The five stimuli were the two low-molecular-weight fractions, the high-molecular-weight fraction, the aqueous earthworm extract, and distilled water. All stimuli except water elicited attacks and increased tongue-flick/attack scores (Figure 1). The ANOVA was highly significant for the stimulus effect ($F = 15.04$, $df = 4, 32$, $P < 0.0001$). Results for the high-molecular-weight fraction

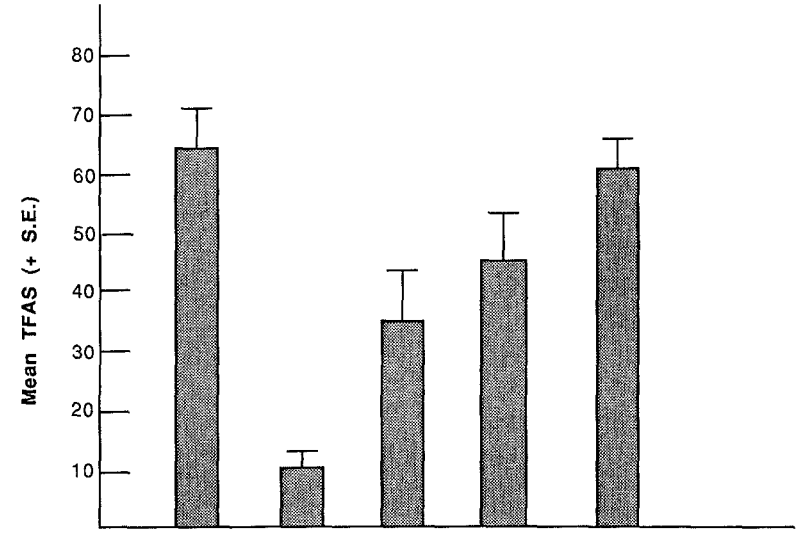


FIG. 1. Mean tongue-flick/attack scores (+1 SEM) and number and mean latency of prey attacks (in 30-sec trials) given by 10 ingestively naive garter snakes to distilled water, aqueous earthworm surface extract, two low-molecular-weight fractions, and a high-molecular-weight fraction prepared from chloroform–methanol earthworm extractions.

were virtually identical to those for the aqueous earthworm extract. Both low-molecular-weight fractions were somewhat less effective than the high-molecular-weight fraction but even the lowest was significantly more effective than water ($Z = 2.45$, $P < 0.01$).

Fathead Minnow Extracts. A Bio-Gel P-10 separation was conducted on the active water layer of the CM extraction. Two fractions were obtained from the preparative P-10 column. A high-molecular-weight fraction eluted at the void volume of the column, and a low-molecular-weight fraction eluted approximately at the volume of the column.

Four snakes were tested once each on four stimuli with test position equalized. The stimuli were the high- and low-molecular-weight fractions, the aqueous minnow fraction, and distilled water. All extracts were significantly more effective than water and statistically indistinguishable from the aqueous extract ($F = 42.18$, $df = 3, 6$, $P = 0.0002$) (Figure 2). The TFAS of the lowest-scoring extract was greater than that for water ($Z = 1.65$, $P = 0.05$).

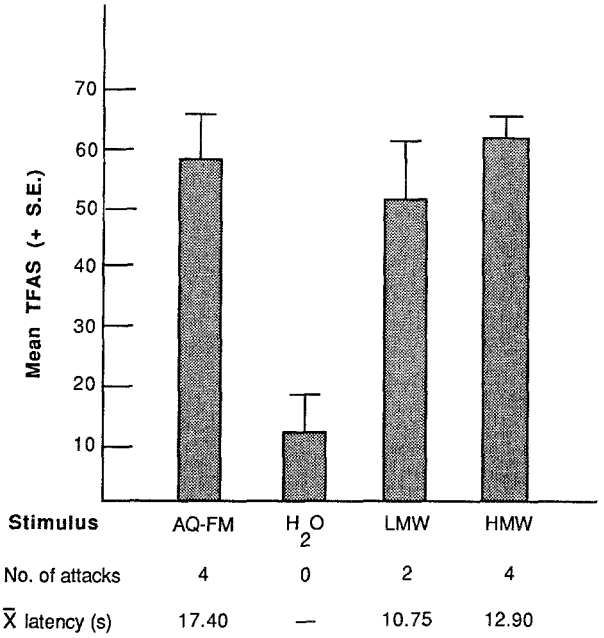
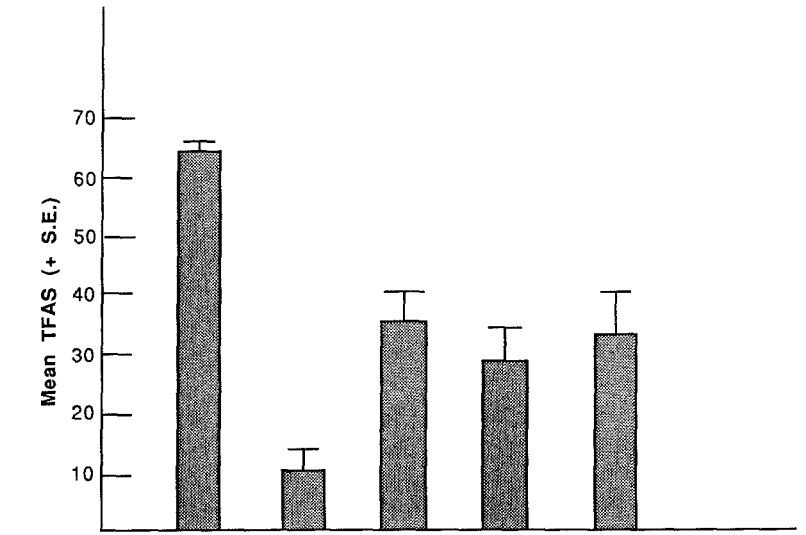


FIG. 2. Mean tongue-flick/attack scores (+1 SEM) and number and mean latency of prey attacks (in 30- sec trials) given by four ingestively naive garter snakes to distilled water, aqueous fathead minnow surface extract, and a low-molecular-weight fraction, and a high-molecular-weight fraction prepared from chloroform–methanol minnow extractions.

All extracts also elicited attacks. Although, as with the earthworm fractions, the low-molecular-weight substances led to a lower TFAS, the low-molecular-weight minnow fraction was, relative to the high-molecular-weight fraction, more effective than the-low molecular-weight earthworm fractions. This confirms the finding with experienced adult snakes reported earlier (Burghardt et al., 1988).

Earthworm Collagen. Ten snakes were tested on five stimuli, as in the earthworm separation experiment. Stimuli tested were collagen prepared according to Muir and Lee (1970), collagen prepared according to Murray et al. (1982) both in a normal and double-concentration reconstitution, aqueous earthworm, and distilled H_2O. TFAS scores varied significantly among stimuli ($F = 26.78$, $df = 4, 32$, $P < 0.0001$). However, while attacks were given to all collagen samples (Figure 3), the TFAS scores were in every case significantly less than scores obtained with the aqueous earthworm extract (Tukey



	AQ-EW	H_2O	Muir & Lee	Murray	Murray (conc.)
No. of attacks	10	0	5	2	4
$\bar{X}$ latency (s)	3.83	—	18.46	4.20	12.45

Fig. 3. Mean tongue-flick/attack scores (+1 SEM) and number and mean latency of prey attacks (in 30-sec trials) given by 10 ingestively naive garter snakes to distilled water, aqueous earthworm surface extract, and three earthworm collagen preparations based on Muir and Lee (1970) and Murray et al. (1982).

HSD test). Even the concentrated Murray fraction was only slightly more effective than the unconcentrated one. This latter extract was the least effective of the collagen fractions but was still more effective than water ($Z = 2.45$, $P < 0.01$).

DISCUSSION

Our previous investigation of earthworm and fathead minnow extracts demonstrated their similar glycopeptide nature and the presence of behaviorally important high- and low-molecular-weight fractions in both extracts (Burghardt et al., 1988). In preliminary investigations it was shown that the high- and low-molecular-weight fractions were significantly different in composition. It was not possible to estimate the absolute purity of the fractions in the earlier study or the relationship of the high- to the low-molecular-weight materials. However, during the separation procedure employed for the present study, a serendipitous problem with the refractive index monitor demonstrated the existence of at least two components in the low-molecular-weight fraction from earthworms.

During separations of several portions of earthworm extract over a two-day period, the response of the detector clearly changed with respect to the low-molecular-weight material. What had appeared as a slightly asymmetrical peak at the start of the separations changed to a partially inverted peak and finally to a completely inverted response that left no doubt that it represented at least two components. During this time the composition of the reference cell solution changed because of ethanol evaporation and thus alerted us to heretofore unrecognized differences in the refractive indices of the low-molecular-weight components. Indeed, when the solution in the reference cell was changed from the 10% ethanol solution normally used for elution of the column to pure water, the observed change in response was reproduced. Collection of fractions when the difference in response was at a maximum provided the two different low-molecular-weight fractions from earthworms employed in the recorded experiments. The overlapping nature of the peaks made complete separation of components impossible, but it was anticipated that a discernible difference in activity of the resulting fractions might be achieved. It was not; both were equally active. Attempts to duplicate this effect with the fish extract were unsuccessful.

The demonstration of responsiveness to the various fractions of earthworm and fish extracts by ingestively naive garter snakes, as well as limited responsiveness to earthworm cuticle collagen material, completes a preliminary study of prey-recognition substances. It is possible that these responses are mediated by similar chemical cues in each of the studied materials. Thus, the high-molecular-weight fractions may be chemically related to the surface collagens of the

prey and, likewise, the low-molecular-weight fractions may be related to the high-molecular-weight materials, presumably as hydrolytic fragments.

The fact that even concentrated earthworm cuticle collagen was less effective than the aqueous extract might either indicate that substances other than collagen are involved or simply reflect the relative insolubility of collagen. In view of the modest responses shown to collagen in this study and our failure to find activity in adult snakes using highly purified collagen (Goss, 1986), it seems likely that collagen activity is due to a substance that is not easily removed during the collagen extraction and purification process. Given the known necessity of a functioning vomeronasal system in the garter snake response to prey chemical cues, this interpretation seems most logical. A small chemically related molecule such as the glycopeptide materials in our readily soluble fractions might be very difficult to remove from the collagen. It could, however, easily be obtained by a tongue-flicking snake and transferred to the vomeronasal organ, a sequence that is less readily imagined for high-molecular-weight collagen molecules. Furthermore, the ability of this snake to discriminate among earthworm and fish species (Burghardt, 1970) argues against the evolution of a chemical prey detection system based solely on the core of a conservative structural macromolecule.

Our previous study demonstrated a dissimilarity of amino acid and carbohydrate composition of the low- and high-molecular-weight fractions from both extracts, and we have now demonstrated the presence of at least two components in one of these fractions. Thus, the composition differences may not reflect dramatically different stimuli. It seems reasonable to suspect that some portion of the glycoprotein is responsible for the activity.

That both of the low-molecular-weight earthworm fractions show activity is not inconsistent with this hypothesis. They could be two related glycopeptide fragments; there could easily be a third component that eluted with both fractions; or overlap of components could have provided activity in both fractions. The manner in which they were separated does not guarantee that separation of either component was complete. The observation of different refractive index responses does not change the obvious tailing of one component into the other; it provides only an arbitrary distinction on which our fractionation was based. Thus, while we failed to demonstrate a distinct difference in biological activity, the fact of chemical difference between the two fractions is certain. A more complete separation of these materials is needed so that their biological relationship can be more convincingly established and their chemical constitutions reliably compared.

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ety of Ichthyologists and Herpetologists, Vancouver, B.C., June 1986. The data on neonate snakes was presented, in part, at the Reptile Chemical Ecology Symposium at the Fifth Annual International Society of Chemical Ecology Meeting, Athens, Georgia, June 1988. Paul Weldon and two reviewers made helpful suggestions on an earlier draft.

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CHEMICAL CUES USED BY PRAIRIE RATTLESNAKES (*Crotalus viridis*) TO FOLLOW TRAILS OF RODENT PREY

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Abstract—Each of 10 prairie rattlesnakes (*Crotalus viridis*) was exposed to three types of trails after striking rodent prey (*Mus musculus*). One trail was made with mouse urine, another was made with tap water, and the third consisted of materials from mouse integument. The snakes exhibited trailing behavior only when integumentary trails were available. It was concluded that prairie rattlesnakes do not utilize urinary cues; instead they attend to materials associated with rodent skin and fur.

Key Words—Rattlesnake, *Crotalus viridis*, mouse, *Mus musculus*, trails, predation.

INTRODUCTION

Prairie rattlesnakes (*Crotalus viridis*) practice several feeding strategies, including active foraging around rodent burrows, sit-and-wait tactics along traditional rodent trails, and carrion eating (Klauber, 1956; Duvall et al., 1985; Diller, 1989; Hennessy and Owings, 1988; see also Gillingham and Baker, 1981; Reinert et al., 1984). When an adult rodent is envenomated, it typically is

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released by the snake within 0.3 sec and allowed to wander while the venom takes effect (Kardong, 1986). A rodent can travel several meters from the site of attack before succumbing to the venom (Estep et al., 1981; Hayes and Galusha, 1984), and the snake then follows the prey's trail in order to recover the carcass (Brock, 1980; Golan et al., 1982; Chiszar et al., 1983, 1986). It has been established that chemical cues are used by the snake during trail-following, but the precise source of these cues remains to be determined (see Burghardt, 1970, for a review of chemical perception in reptiles; see also Kubie and Halpern, 1979; Halpern and Kubie, 1980).

It has long been our suspicion that rodent urine contains the cues used by rattlesnakes. This idea was based partly on the fact that mice usually urinate copiously upon being envenomated and partly on the fact that rodent urine contains a variety of chemicals known to be important for rodent communication, social behavior, reproduction, and development (Vandenberg et al., 1976; Drickamer, 1977; Bronson, 1979; Nyby, 1983). Consequently, we speculated that rattlesnakes detect some of these chemicals, especially urinary components that might be associated with alarm. Rodents also possess several glands that produce materials that are spread on their integuments through grooming. Such materials might adhere to rodent paws and therefore be deposited on the substrate as an envenomated individual wanders away from the site of attack.

The present experiment was designed to determine whether trail cues are of urinary or integumentary origin. Although we fully expected prairie rattlesnakes to follow urine trails, a pilot study (Duvall and Chiszar, 1989) showed that such trails were ignored and that snakes followed only trails made with integumentary materials. The present study was therefore conducted to replicate the surprising results of the pilot study.

We are mindful of the possibility that some alarm substances produced by mice might be nonurinary and might, therefore, be part of the integumentary materials that we manipulate in this study. Hence, we do not view the distinction between urinary and integumentary trails as being correlated with presence versus absence of alarm substances. See Thiessen and Cocke (1986) for a review and conceptualization of rodent alarm substances, and see Weldon (1983) for related evolutionary considerations.

METHODS AND MATERIALS

Subjects. Ten adult *C. viridis* were observed (five others had been observed by Duvall and Chiszar, 1989). These snakes had been in captivity for at least three years, living in individual glass cages (50 × 27.5 × 30 cm) and eating one mouse (*Mus musculus*) weekly. Although the snakes had been used in previous experiments involving presentation of chemical cues, no invasive manip-

ulations had been performed. Hence, these snakes were typical of long-term captives.

Procedure. Each snake was observed in four experimental conditions, and all of these observations were made in wooden pens (180 × 66 × 81 cm) divided into two compartments (150 and 30 cm long, respectively) by a partition containing a guillotine door. The smaller compartment contained a paper floor cover and a stainless-steel vessel filled with water. A snake was placed into this compartment for one week before the start of observations, which were then made at the rate of one per week for four weeks. See Golan et al. (1982) for a description of laboratory temperature, photoperiod, and other conditions. Three identical pens were used simultaneously in this study.

A paper covered the floor of the large compartment, and a solid meandering line (150 cm) was drawn on the paper, starting from the guillotine door and ending behind a rock on the opposite side. Two additional dashed lines paralleled the first one, each 2 cm from the solid line. Trails were deposited along the center line, and the space between the two dashed lines constituted the trail area. A snake was judged to be in the trail area if its head was between the dashed lines. The trail area comprised 11.3% of the floor area in the large compartment.

In three of the experimental conditions, snakes struck a mouse in the small compartment just prior to the opening of the guillotine door, permitting them to enter the large compartment. This mouse was suspended in the small compartment by forceps and was removed immediately after the strike. Hence, the mouse never touched the floor or walls of the small compartment, and deposition of odoriferous materials was therefore minimized. In the large compartment, one of three trails was available: urine, water, or integumentary material.

Mouse urine was collected in metabolism cages and stored at 13°C until needed but never for more than 48 hr. Urine was then warmed to 35°C and pipetted onto the trail line at the rate of one drop per 5 cm. This was done 2.5 min prior to permitting the snake to strike a mouse and beginning a trial. Urine drops usually expanded to form a circle of 1–2 cm diameter. So, for a control (i.e., blank) trail, we pipetted tap water along the center line at the rate of one drop per 5 cm. The integumentary trail was deposited by dragging a freshly killed mouse carcass along the center line. The inguinal region of this carcass was covered with cotton, and the carcass was dragged in dorsal recumbancy. No urine was visible on this trail, and we suspect that the only chemicals deposited were those that are normally present on the fur.

A freshly killed mouse carcass was positioned behind the rock at the end of the trail. This carcass was not used to make integumentary trails, nor was it a urine donor. Dependent variables were: (1) rate of tongue-flicking per minute (RTF) during 5 min prior to the predatory strike (i.e., baseline RTF), (2) RTF following the strike, (3) latency to make initial contact with the trail area, (4)

number of seconds that the snakes' heads were in the trail area, (5) number of tongue-flicks emitted while the head was in the trail area, and (6) latency to discover the carcass at the end of the trail (timed from the moment snake made initial contact with trail area until the snake touched the carcass). A maximum of 20 min was allowed per trial. If a snake did not reach the end of the trail during this time, a score of 1200 sec was assigned.

The fourth observation involved a no-strike presentation of a mouse at the start of the trial. A live mouse was suspended in the small compartment, but was held out of striking range for 5 sec and then removed. An integumentary trail had been deposited in the large compartment, and a freshly killed mouse was behind the rock. The guillotine door was raised immediately after the live mouse was withdrawn from the small compartment. Dependent variables were the same as those taken after strikes had been delivered.

Each snake was observed in all conditions, but a different random order of trials was used for each snake. Paper floor coverings were removed from the large compartment after each trial and replaced with clean ones.

Snakes were permitted to eat the carcass at the end of the trail upon discovering it. If this carcass was not discovered within 20 min, it was moved by forceps to the snake's mouth and ingestion was permitted. If a snake refused food, the data from that day were discarded, and food was again offered three days later. Trials were resumed seven days after the snake ate. This occurred only three times. No trials were run if a snake exhibited signs of ecdysis, but they were resumed seven days after the first postecdysis feeding. This occurred twice. Hence, each snake ate after each trial from which data are reported, and hunger was thereby maintained at a constant level across the four trials (i.e., snakes were always seven days hungry at the start of a trial).

RESULTS

Since all snakes were observed in all experimental conditions, data were analyzed by repeated-measures analyses of variance (ANOVAs) containing three factors: subjects, conditions, and subjects × conditions (i.e., the appropriate denominator for F ratios testing the main effect of conditions) (Winer, 1971). In most ANOVAs the respective degrees of freedom were 9, 3, and 27, with 3 and 27 being associated with F ratios (Table 1). When subsets of conditions were selected for analysis, the degrees of freedom associated with conditions and subjects × conditions were adjusted accordingly.

Average RTF during baseline observations was 2.7, and the four conditions did not differ ($F < 1$), indicating that snakes were quiescent prior to introducing live mice into the small compartment. Mean RTF during the three strike trials (i.e., from the strike until the trial terminated) was 39.5 and the

TABLE 1. MEANS OF FOUR DEPENDENT VARIABLES IN EACH EXPERIMENTAL CONDITION[a]

Dependent variable	Condition				Average SEM[b]	F 3/27
	No strike, integumentary trail	Strike, urine trail	Strike, water trail	Strike, integumentary trail		
Mean latency to contact trail area (sec)	736.0a	262.4b	288.5b	225.6b	101.1	3.79[c]
Mean % time on trail	1.9a (−1.50)	10.1a (−0.19)	7.7a (−0.57)	33.7b (3.58[c])	6.2	31.30[c]
Mean % tongue flicks on trail	3.8a (−1.37)	12.5a (0.21)	7.4a (−0.71)	35.0b (4.33[c])	5.4	33.50[c]
Mean latency to reach carcass (sec)	976.1a	642.5b	602.7b	485.5c	53.2	3.72[c]

[a] Means within a row bearing the same letter did not differ significantly by post hoc tests (Newman-Keuls). Values in parentheses are single-sample t tests ($df = 9$) comparing tabled mean with expected score of 11.3 (see text).

[b] The Greenhouse-Geisser (1959) test confirmed equality of variances and covariances across experimental conditions for each measure. Hence, average SEMs are representative of standard errors within each condition.

[c] $P < 0.05$.

three types of trials did not differ ($F < 1$). Mean RTF during the no-strike trial was 22.2. After presentation of the mouse, RTFs were higher than the baseline rate ($F = 134.57$, $df = \frac{1}{9}$, $P < 0.05$), and the mean poststrike RTF was significantly higher than the mean RTF after no-strike presentations of mice ($F = 5.39$, $df = \frac{3}{27}$, $P < 0.05$).

The remaining dependent variables are summarized in Table 1. After striking a mouse, snakes typically made contact with the trail area in less than 5 min, and this measure did not vary as a function of type of trail. After no-strike presentations, however, there was a much longer latency to contact the trail. In fact, only four of 10 snakes passed through the guillotine door and contacted the trail area after no-strike presentations, while there was only a single failure to contact the trail in all of the strike conditions combined. Three sign tests compared this aspect of performance after no-strike presentations to that after each of the three strike presentations ($Ps = 0.03$, 0.03, and 0.06). These data plus the RTF data suggest that striking activates searching behavior and that chemical cues in the environment make no additional contribution to this initial aspect of poststrike performance (Golan et al., 1982; Chiszar, 1986).

The remaining rows of Table 1, however, reveal that behavior following initial contact with the trail was strongly influenced by the chemical contents of the trail area. Since no-strike presentations did not inspire sustained searching behavior, the trailing scores were very low in this condition. This result confirms previous findings (Golan et al., 1982; Chiszar et al., 1983), and the no-strike performance will not be discussed further. The only strike condition that resulted in significant trail-following was the one containing an integumentary trail. This can be seen in two ways in the second row of Table 1. First, the mean percent time on trail was significantly higher in the strike-integumentary trail condition than in the other two strike conditions. Second, the *t* tests reported in Table 1 compare the mean percent time on trail with the chance expectation of 11.3%. Since the trail area occupied 11.3% of the floor area in the large compartment, a snake moving randomly in that compartment would be expected to have its head in the trail area about 11.3% of the time. Only in the strike-integumentary trail condition did the mean percent of time on trail exceed 11.3%. The same pattern of results also obtained for the percent tongue-flicks on trail. Indeed, all 10 snakes exhibited trailing behavior by both measures (i.e., scores greater than 11.3%) when an integumentary trail was available; none did so with a water trail, and only one did so with a urine trail.

Not surprisingly, snakes discovered the hidden carcasses more rapidly in the strike-integumentary trail condition than in all other conditions. Indeed, we suspect that the differences seen with this measure would be much larger if a larger apparatus was used for trail-following tests because the probability of a randomly moving snake discovering the carcass by chance would be lower than was the case in our relatively small pen. This would increase the difference between performance in the strike-integumentary trail condition and that seen in the other strike conditions. Nevertheless, a prairie rattlesnake that struck a mouse and found an integumentary trail discovered the hidden carcass in our apparatus in 25% less time than was needed when no trail or a urine trail was available.

DISCUSSION

These results and those of Duvall and Chiszar (1989) indicate that trails made with mouse urine are not used by prairie rattlesnakes in laboratory tests. The active materials seem to be chemicals associated with mouse integument. Since chemicals from many exocrine glands and other sources contribute to skin and fur chemicals, it is premature to speculate about the particular components that rattlesnakes use during trail-following (but see Blake et al., 1983). Furthermore, it is not necessarily the case that endogenously generated compounds are exclusively used, since Melcer and Chiszar (1989) have shown that exog-

enous materials applied to mouse integuments can be detected by rattlesnakes during predatory strikes and remembered for at least 60 min afterwards. Consequently, the most reasonable conclusion at present is that rattlesnakes obtain information from rodent integument. Some integumentary contents are probably deposited (by rodent paws) during locomotion, and rattlesnakes apparently have an ability to detect and follow such trails.

Desert and prairie rodents urinate much less than do laboratory strains of *Mus musculus*. Perhaps rattlesnake attention is focused on nonurinary chemicals because urinary ones are less available and, therefore, less dependable under natural conditions (but see Cushing, 1984).

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CHEMICAL AND BEHAVIORAL ECOLOGY OF FORAGING IN PRAIRIE RATTLESNAKES (*Crotalus viridis viridis*)

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Abstract—Free-ranging prairie rattlesnakes (*Crotalus viridis viridis*) exhibit lengthy vernal migrations upon emergence from winter hibernation. A series of laboratory experiments was designed to test hypotheses regarding the function and causation of vernal movements. Rattlesnakes obtained from Wyoming and Colorado populations were used. First, we hypothesized that the function of vernal movements is to locate small mammal prey. Second, we predicted that active *C. v. viridis* use prey chemicals, as well as other cues, to decide whether or not rodents are present in an area. Third, we hypothesized that vernally active males would be more responsive to rodent prey and their odors than females, given observed differences in behavior in the field. Fourth, we predicted that rattlesnakes captured in Colorado would be more sensitive to prey odors than those obtained in Wyoming, because of disparate community structure and, hence, small mammal spatial distributions. As expected, snakes exhibited reduced activity, as well as certain other dependent measures reflecting predatory investigation, in arena zones containing either live rodents or their chemicals. However, responses to the latter were reduced in Wyoming rattlesnakes tested with chemicals from deer mice (*Peromyscus maniculatus*), relative to Colorado animals tested with chemicals obtained from house mice (*Mus musculus*). In contrast to patterns observed in nature, males and females exhibited almost no differences in overall responsiveness. Results are discussed in the context of simulation modeling and ongoing studies of prairie rattlesnake behavior.

Key Words—*Crotalus viridis viridis*, rattlesnake, foraging, predation, chemical signals.

INTRODUCTION

Prairie rattlesnakes (*Crotalus viridis viridis*) in southcentral Wyoming exhibit lengthy, straight-line (i.e., fixed angle) vernal migrations (Duvall et al., 1985; King, 1987). Males travel on straighter lines over longer distances than do females (King and Duvall, 1990). This minimizes the likelihood that males and, to a lesser extent, females will cover the same terrain over again, as they search for food and other resources. Prairie rattlesnakes probably are not unique among snakes in these respects (Gibbons and Semlitsch, 1987; Gregory et al., 1987; Macartney et al., 1988).

Field data indicate that the primary function of the vernal phase of the season-long migration among *C. v. viridis* is to find food (Duvall et al., 1985; King, 1987; King and Duvall, 1990). Field experiments corroborate this view (Duvall and Chiszar, 1989; Duvall et al., unpublished data). At our field site in the high desert communities along the Continental Divide of southern Wyoming, *C. v. viridis* eat primarily deer mice (*Peromyscus maniculatus*), the most common small mammal in the area. Deer mouse populations are widely and patchily distributed, and their occurrence is unpredictable from year to year (Duvall et al., 1985; King, 1987). This circumstance poses a significant foraging problem for the rattlesnakes, especially given their brief active or growing season (ca. 95 days/year; King and Duvall, 1990). Males must also search for receptive females as the summer progresses, compounding the importance of their finding food early in the season (King and Duvall, 1990).

We have hypothesized that the survival value of lengthy, straight-line vernal movements lies in the search for spatially unpredictable and widely distributed small mammal prey. In field research conducted from 1982 to 1984, we found that migrating snakes ceased movement upon initial encounters with patches of deer mice (Duvall et al., 1985; King, 1987; King and Duvall, 1990). Moreover, because males face the dual problems of finding food and mates (females concentrate only on the former; King and Duvall, 1990; Trivers, 1972), we have hypothesized that they would be especially responsive to deer mouse patches located in the spring, and relatively more responsive to these rodents and stimuli derived from them than are females. This is because females feed all summer long, while males feed primarily in the first half of the season (King and Duvall, 1990). Males are unresponsive to food and highly responsive to females during the latter half of the summer.

Given the demonstrated importance of the chemical senses to the behavior of snakes and lizards (e.g., Bellairs, 1984; Burghardt, 1970; Halpern and Kubie, 1984), we hypothesized that migrating (= active or locomoting in a lab setting; see below) male more so than female *C. v. viridis* would employ prey chemicals to determine if rodents occur in particular areas. Prairie rattlesnakes that have

located rodent patches in the field exhibit predatory actions ranging from active searching, characterized by reduced locomotory rates and elevated tongue-flicking, to carrion feeding, to sit-and-wait ambushing (Duvall et al., unpublished data; cf., Diller, 1989; Gillingham and Baker, 1981; Gillingham and Clark, 1981; Golan et al., 1982; Hennessy and Owings, 1988). Hence, dependent measures reflecting this variation in predatory response were employed here (see below).

We also compared responses of snakes from different populations. Work by Arnold (1981) and Drummond and Burghardt (1983) has shown that different populations of garter snakes (*Thamnophis* spp.), occupying disparate local selection regimes, exhibit qualitatively different patterns of behavior. Even though we know very little about the behavior of *C. v. viridis* in the spatially homogenous, Pawnee grassland vegetation communities of northeastern Colorado, we attempted to compare snakes from this population with those from the Wyoming site. Differences in key habitat parameters (e.g., summer seasons are about two months longer on the Pawnee grasslands; Martner, 1986) and community structure (e.g., uniform spatial distribution of dominant grasses could in turn lead to a uniform spatial distribution of rodents) between the two sites may have resulted in the evolution of differing patterns of foraging behavior, including functional reasons for vernal migrations and responses to prey chemicals.

The work discussed here was designed to test the following hypotheses: (1) one function of vernal movements of *C. v. viridis* is to find food, as evidenced by reduced activity in and preferences for regions of test arenas containing live prey or certain prey-derived stimuli; (2) prey chemicals alone can signal the presence of rodent prey in a test arena zone to a locomoting *C. v. viridis*; (3) males from the Wyoming site would be more responsive to prey and prey-derived stimuli than are females, when tested in spring and early summer; and (4) subjects drawn from two allopatric populations, occurring in regions with different habitats and communities, would respond differently to chemicals derived from rodents.

METHODS AND MATERIALS

Four experiments are described. The first three were conducted at the University of Wyoming, Laramie, and the fourth at the University of Colorado, Boulder. Although the objectives of the four experiments were similar, different designs were used in the two laboratories. Both general and specific methods sections are presented for experiments 1–3. One comprehensive methods section is given for experiment 4.

General Methods in Wyoming Experiments

Subjects and Maintenance. Twenty-eight *C. v. viridis* were captured as they emerged from hibernation in the spring and were transported immediately to Laramie. Half were males, half were nonpregnant females. [Females eat little or nothing in the seasons they are pregnant and, hence, are not discussed here (see Duvall et al., 1985; Graves et al., 1986).] The dens from which snakes emerged are in the Haystack Mountains of the northeastern Red Desert, along the Continental Divide of southcentral Wyoming. Snakes were housed and maintained between trials in glass aquaria fitted with clean paper flooring, a hide box, a water bowl, and a 100-W heat lamp. All subjects were maintained and tested on a 14:10 light–dark photoperiod. Ambient temperature averaged 23.6°C (range = 19.4–27.8°C) throughout maintenance and experimentation. All three experiments were conducted in late spring and summer.

Deer mice, the preferred prey of snakes at our Wyoming field site (King, 1987), were live-trapped and brought to Laramie both to maintain snakes and to use in experiments. Deer mice were provided commercial wild bird seed mix and water ad libitum. To determine if subjects would be responsive to prey in our protocol, snakes were offered at least one live deer mouse (*Peromyscus maniculatus*) prior to experiments. Snakes that did not readily attack and accept prey, and those shedding, were excluded from the study.

Experimental Design, Testing, and Analytic Procedures. Behavioral tests took place in wooden arenas (240, 76, and 90 cm in length, width, and height, respectively), the floor of which was covered with a fresh piece of Kraft food wrapping paper prior to each trial. The paper on the floor was demarcated with a pen into two equal sized halves (76 × 120 cm). Next, a smaller rectangular area (34.5 × 45 cm) was drawn in the center of the floor of each of the respective halves, in which one white plastic mouse cage (29.5 × 18.5 × 13 cm) was then centered. Depending on the experiment, mouse cages (i.e., prey conditions) contained either (1) clean sawdust shavings, (2) shavings soiled by deer mice for a period of two weeks, or (3) similarly soiled shavings plus two live deer mice. Arena sides containing respective conditions were randomly determined prior to each test trial. Mouse cages were covered with hardware cloth tops and fitted with water bottles. A water bowl for snakes during trials was placed along the midline of arenas.

Trials commenced as individual subjects were lowered by snake hook onto the center of the arena floor. Snakes were then observed during nine, 10-min intervals spread over a period of three days. Observations occurred between 0630 and 0830, 1230 and 1430, and 1800 and 2000 hr, on the first, second, and third days.

Dependent variables quantified during trials included (1) percent time spent on either side of the midline, (2) percent time spent in the rectangular area surrounding respective cages/conditions, divided by percent duration on a par-

ticular side, (3) percent time spent in ambush postures within respective rectangular areas (characterized by snakes lying coiled with the snout oriented towards the stimulus, the neck retracted slightly, and the head lying on the cage; see Reinert et al., 1984), divided by duration in respective rectangular arenas, (4) accumulated tongue-flicks on the respective sides, (5) accumulated mouth gapes on the respective sides (see Graves and Duvall, 1983), (6) accumulated peers (where snakes tilted their heads to inspect cage contents from above) on the respective sides, (7) accumulated nudges (where snakes pushed against the cage with their snout) on the respective sides, and (8) accumulated strikes (where snakes bit at the contents of a cage from above) on the respective sides. All proportional data were arcsin transformed prior to ANOVA.

Experiments 1, 2, and 3 corresponded to a $2 \times 2 \times 9$ (prey conditions by sex of snake by observation trials), mixed repeated measures analysis of variance (ANOVA) design. Therefore, prey conditions were presented to males and females as a simultaneous two-choice discrimination factor.

Experiment 1

We have observed free-ranging *C. v. viridis* investigating rodent burrows in the field with high levels of tongue-flicking, as well as establishing ambush sites in active deer mouse patches where thermal or visual cues derived from prey are not always immediately available (Duvall et al., 1985, unpublished data; King, 1987; cf., Klauber, 1972; Reinert et al., 1984). We hypothesized that snakes rely on the presence of deer mouse odors in an area to make decisions about when to cease migrating and where to search or ambush and that males more than females would prefer to occupy areas containing prey chemicals.

Specific Methods. The 11 males and 11 females used in this experiment were exposed to a 2-cm-deep layer of clean shavings in the bottom of one mouse cage and a layer of soiled deer mouse shavings of similar depth in the other. However, because of ecdysis, only eight males and seven females concluded the experiment and comprised the sample analyzed.

Results. Responses of snakes exposed to cages containing clean and deer mouse shavings are presented in Table 1. No significant main or interaction effects were detected.

Experiment 2

Specific Methods. This study was similar in design and purpose to experiment 1, except that the 2-cm-deep layers of clean or soiled shavings were elevated by a false bottom in mouse cages up to and flush with hardware cloth tops. This allowed snakes to make lingual contact with any prey chemicals in

TABLE 1. RESPONSES ($\bar{X} \pm$ SE) OF *C. v. viridis* TO DEER MOUSE-SOILED AND UNSOILED SHAVINGS[a]

		Dependent measures[b]							
Condition	Sex	T_{side}	$T_{rectangle}$	T_{ambush}	Tongue flicks	Gapes	Peers	Nudges	Strikes
Deer mouse shavings	Males	44.3 ± 5.5	10.3 ± 3.4	5.8 ± 2.7	13.3 ± 2.9	0.0	0.0	0.0	0.0
	Females	46.7 ± 6.3	21.3 ± 5.0	15.9 ± 4.6	12.1 ± 4.6	0.0	0.1 ± 0.04	0.2 ± 0.2	0.0
Unsoiled shavings	Males	56.5 ± 5.5	25.8 ± 4.9	20.0 ± 4.7	26.7 ± 8.7	0.0	0.1 ± 0.04	0.1 ± 0.03	0.0
	Females	53.4 ± 6.3	13.4 ± 4.2	8.4 ± 3.4	17.0 ± 6.7	0.0	0.0	0.0	0.0

[a] Eight males and seven females tested. All proportional data arcsin transformed prior to analysis of variance. See text for discussions of statistical significance.

[b] T_{side} = percent time spent on respective sides of the arena midline; $T_{rectangle}$ = percent time spent in rectangular areas surrounding respective cages/conditions, divided by the percent duration spent on a particular side; T_{ambush} = percent time spent in ambush postures (see text for explanation) within respective rectangular areas, divided by respective $T_{rectangle}$. See text for explanations of all additional dependent measures.

the shavings, presumably facilitating vomeronasal organ (VNO) chemosensory investigation (e.g., Bellairs, 1984). The design employed in experiment 1 could not guarantee this and may have partly resulted in the absence of significant effects. It seemed possible that key prey-derived exudates might be nonvolatile chemicals requiring direct physical contact with the tongue, in order to make their way into the VNO (e.g., Kubie and Halpern, 1978). Twelve males and nine females were used in the experiment.

Results. Descriptive statistics reflecting the responses of snakes to clean versus soiled shavings that were moved by a false bottom up near the tops of cages are presented in Table 2. In contrast to experiment 1, a main effect was detected; both males and females exhibited significantly more time in ambush postures (T_{ambush}) near cages containing soiled as opposed to clean shavings [$F(1, 19) = 4.321$, $P < 0.049$].

Experiment 3

Only one of several dependent measures was significant in experiment 2, and none were in experiment 1. Hence, we must interpret an apparent VNO-mediated T_{ambush} effect cautiously. Although most of the data argue against such a conclusion, it appears that snakes from Wyoming are guided to some extent by prey chemicals. However, since migrating snakes in nature probably detect prey stimuli other than just chemical ones in making decisions about when and where to cease moving and begin to forage (e.g., Hennessy and Owings, 1988), we hypothesized that snakes would be responsive to a more complete configuration of prey stimuli rather than to chemical cues alone. The paucity of major effects in experiments 1 and 2 seems consistent with this observation. Furthermore since *C. v. viridis* at the Wyoming site forage in a community where deer mice are widely distributed and spatially unpredictable, we hypothesized that active rattlesnakes studied in the laboratory would be more responsive to actual mice than any potentially indirect (e.g., chemical) evidence of their physical presence. Again, we also expected greater responsiveness by males.

Specific Methods. Snakes in this experiment also were exposed to two cages, one containing two live deer mice residing on a 2-cm-deep layer of soiled shavings and the other a 2-cm-deep layer of soiled shavings alone. Eleven males and nine females were used in this experiment.

Results. Responses of snakes to cages containing soiled shavings or live deer mice residing on soiled shavings are presented in Table 3. Unlike experiment 1 and 2, the conditions factor resulted in a number of significant main effects. Both males and females spent significantly more time on the side (T_{side}), time in the rectangle ($T_{rectangle}$), T_{ambush}, and exhibited more peering (peers), [$F(1, 18) = 10.033$, $P < 0.005$; $F(1, 18) = 15{,}869$, $P < 0.001$; $F(1, 18) = 13.141$, $P < 0.002$; and $F(1, 18) = 5.952$, $P < 0.024$, respectively] in arena

TABLE 2. RESPONSES ($\bar{X} \pm$ SE) OF *C. v. viridis* TO DEER MOUSE-SOILED AND UNSOILED SHAVINGS POSITIONED TO ALLOW DIRECT TONGUE–VNO CONTACT[a]

		Dependent measures[b]							
Condition	Sex	T_{side}	$T_{rectangle}$	T_{ambush}[c]	Tongue flicks	Gapes	Peers	Nudges	Strikes
Deer mouse shavings	Males	52.2 ± 4.6	29.0 ± 4.2	23.3 ± 4.1	26.2 ± 6.5	0.0	0.0	0.0	0.0
	Females	51.6 ± 5.5	24.5 ± 4.7	15.5 ± 4.0	12.5 ± 4.4	0.0	0.0	0.0	0.0
Unsoiled shavings	Males	47.8 ± 4.6	14.3 ± 3.0	3.7 ± 1.8	42.2 ± 8.7	0.0	0.0	0.0	0.0
	Females	48.4 ± 5.5	13.9 ± 3.8	7.4 ± 2.9	8.7 ± 2.2	0.0	0.0	0.0	0.0

[a] Twelve males and nine females tested. All proportional data arcsin transformed prior to analysis of variance.
[b] See footnote *b*, Table 1, and text for explanations of dependent measures.
[c] Significant main effect; see text for discussions of statistical significance.

TABLE 3. RESPONSES ($\bar{X} \pm$ SE) OF *C. v. viridis* TO LIVE DEER MICE AND DEER MOUSE-SOILED SHAVINGS[a]

		Dependent Measures[b]							
Condition	Sex	T_{side}[c]	$T_{rectangle}$[c]	T_{ambush}[c]	Tongue flicks	Gapes	Peers[c]	Nudges	Strikes
Live deer mice	Males	64.5 ± 4.7	45.1 ± 4.8	34.2 ± 4.7	27.3 ± 10.0	0.0	0.6 ± 0.3	0.4 ± 0.1	0.7 ± 0.5
	Females	67.7 ± 4.6	44.9 ± 5.4	37.6 ± 5.3	25.3 ± 5.4	0.0	0.3 ± 0.1	0.2 ± 0.1	0.0
Deer mouse shavings	Males	35.3 ± 4.7	11.5 ± 3.0	9.0 + 2.9	22.3 ± 7.7	0.0	0.1 ± 0.1	0.2 ± 0.1	0.0
	Females	32.4 ± 4.6	12.9 ± 3.7	3.7 ± 2.1	16.0 ± 4.2	0.0	0.0	0.0	0.0

[a] Eleven males and nine females tested. All proportional data arcsin transformed prior to analysis of variance.
[b] See footnote *b*, Table 1, and text for explanations of dependent measures.
[c] Significant main effect; see text for discussions of statistical significance.

halves containing caged deer mice. These main effects were paralleled by significant two-way interactions for conditions by trials for T_{side}, $T_{rectangle}$, and T_{ambush} [$F(8, 144) = 2.439$, $P < 0.017$; $F(8, 144) = 2.339$, $P < 0.021$; and $F(8, 144) = 2.258$, $P < 0.026$, respectively], indicating that snakes came to prefer arena zones containing live deer mice as trials progressed. A significant two-way interaction for sex by trials for nudges [$F(8, 144) = 2.464$, $P < 0.016$] indicates that males but not females exhibited a decrease in this action across trials. No additional main or interaction effects were detected.

Experiment 4

Evidence suggests that snakes vary in the extent, duration, directionality, and timing of seasonal patterns of activity (e.g., Gibbons and Semlitsch, 1987; Gregory et al., 1987; Macartney et al., 1988). We have argued that the length, duration, timing, and so forth, of vernal and seasonal movements exhibited by *C. v. viridis* may vary between populations as a function of local community and habitat selection variables (Duvall et al., 1985; King and Duvall, 1990). Individuals residing in the grasslands of northeastern Colorado—where rodents might be expected to be more uniformly distributed than at our Wyoming site, because of a more uniform spatial distribution of locally dominant grasses—might be just as likely to exhibit a random walk while foraging than the lengthy straight-line movements of snakes living in the patchy and unpredictable prey communities characteristic of the Wyoming site. In models where prey resources are widely or patchily distributed, computer simulations of search paths for *C. v. viridis* foragers indicate that the most spatially efficient route is a straight line (Duvall et al., unpublished data). That is, straight line paths give the highest probability of patch location. However, when resource units are more uniformly distributed and closer together, there is no benefit to traveling along straight-line or fixed-angle paths, as opposed to random walks. Hence, it is in the latter type of community that *C. v. viridis* should be expected exclusively to sit and wait in ambush for prey.

What this means in the context of the work discussed here is that Colorado snakes may be more responsive to chemicals derived from potential rodent prey than are Wyoming animals, because waiting in ambush for prey, as opposed to actively searching for them, has a better chance of paying off in a community where prey resources are less widely distributed in space. Accordingly, we hypothesized that when tested in the laboratory, rattlesnakes from the grasslands of northeastern Colorado would be highly responsive to arena zones containing chemicals derived from rodent prey.

Maintenance and Source of Subjects. Five long-term resident *C. v. viridis*, captured in the Pawnee grasslands of northeastern Colorado, comprised the experimental subjects in this study. The experiment was conducted at the Uni-

versity of Colorado, Boulder, and also is discussed by Duvall and Chiszar (1989). These individuals were maintained on a diet of house mice (*Mus musculus*).

Procedures. This experiment was designed to manipulate the presence versus absence of house mouse chemicals in a 3 × 5-m seminatural indoor arena. Sex of snakes was not an independent variable in this experiment. The five snakes studied were individually placed into the enclosure and left there for two weeks. No prey odors were presented during this period. The floor of the arena was covered with a 4-cm-deep layer of dirt and gravel, and large rocks were distributed randomly throughout. A glass water bowl was centrally located. Ambient temperature in the arena was maintained at ca. 26°C by an electric heater and a 12 : 12 light–dark photoperiod was employed. The floor of the arena was marked so that 15, 1 × 1-m square zones could be distinguished. Records of particular squares occupied by snakes were made.

On days 15–20, two steel mouse cages with screen tops were placed into the enclosure. Each cage was placed into a randomly selected square. One of these contained clean shavings, the other shavings that had been soiled by house mice for one week. No mice were present in either container when placed into the arena. Squares occupied by snakes were recorded 10 times a day, for six consecutive days.

During the first two weeks snakes moved more or less randomly in the arena. There was a slight preference for corner squares, but each snake was observed to move throughout the arena and to visit the water bowl and most of the squares. After the third day, the rate of movement declined, but there remained a tendency for the snakes to make excursions through the arena. Since corner squares were preferred, the cages containing shavings were never placed in corners. This was the only restriction on the otherwise random selection of sites for cages.

When the clean and soiled containers were in place (days 15–20), it was observed that snakes initially positioned themselves next to or on top of the container with soiled shavings. This behavior disappeared over succeeding days, so data for days 15–17 and 18–20 were analyzed separately. We tallied all observations during which each snake was in the square containing soiled shavings, clean shavings, and all other squares. These tallies were converted to percents and averaged over the five snakes. Single sample t tests were used to analyze spatial preferences of snakes during days 15–17 and days 18–20.

Results. Descriptive statistics reflecting snakes' spatial preferences are presented in Table 4. An alpha of 0.05 was used as the acceptance level for all significant effects reported for experiment 4. During days 15–17, snakes were found more frequently than expected near the soiled cage [$t(4) = 11.30$], and less frequently than expected near the clean cage [$t(4) = -10.00$] and all other squares [$t(4) = -9.76$]. Since the snakes had a slight preference for corner

TABLE 4. PERCENT ($\bar{X} \pm$ SE) PREFERENCES OF *C. v. viridis* FOR TEST ARENA REGIONS CONTAINING HOUSE MOUSE-SOILED, UNSOILED, OR NO SHAVINGS[a]

	Preference for:		
Days	Soiled squares	Unsoiled squares	All other squares
15–17	70.0 ± 5.6[b] (6.7)	0.7 ± 0.6[b] (6.7)	29.1 ± 5.9[b] (86.7)
18–20	12.9 ± 7.0 (6.7)	4.1 ± 1.5 (6.7)	83.0 ± 12.2 (86.7)

[a] Expected percent spatial preference scores in parentheses. This experiment also is discussed by Duvall and Chiszar (1989).
[b] Deviates significantly from expected. See text for discussions of statistical significance.

squares during days 1–14, it is all the more interesting that soiled shavings located in noncorner squares were successful in attracting snakes during days 15–17. During days 18–20, however, snakes were no longer attracted to the soiled cages. Spatial preferences for clean and all other squares likewise did not differ significantly from expected. Finally, a paired t test revealed a significant reduction in mean percent attendance at the soiled container between days 15–17 and 18–20 [$t(4) = 4.58$].

DISCUSSION

As expected, *C. v. viridis* tested in the laboratory were attentive to prey-derived stimuli, and they reduced activity and came to reside in arena zones containing such cues. In contrast to behavior observed in the field (Duvall and Chiszar, 1989; King and Duvall, 1990), however, there were almost no differences detected between males and females in the laboratory experiments reported here. Whether mediated by chemical or other cues, the results are consistent with the functional hypothesis that the survival value of vernal migrations in nature is to find prey. Although this strictly applies only to the Wyoming snakes, since they had recently emerged from hibernation when tested, we expect the same for northeastern Colorado snakes in nature. In Wyoming, for example, we know that the major alternative functional explanations for vernal movements—finding mates and appropriate microrefugia for ecdysis—do not operate until much later in the season (Duvall et al., 1985; King, 1987; King and Duvall, 1990). Finding prey always precedes mating and almost always precedes shedding at the Wyoming site, even though intermittent but reduced feeding, especially among females, may continue throughout the active season (King and Duvall, 1990). Perhaps the sequence of survival "problem solving" exhibited

by Colorado snakes is similar. However, because of differences in community structure and key habitat variables in Colorado as opposed to Wyoming (e.g., a potentially more uniformly distributed prey base, longer growing seasons), it is possible that the sequence in which snakes confront the problems of finding food, mates, shedding sites, and so forth, may be more variable. The observation by Klauber (1936) that females in eastern Colorado can show annual reproductive cycles, supports the supposition that greater prey availability and increased seasonal warmth occur there (cf., Diller and Wallace, 1984).

Responses to prey chemicals were variable and complex. Preferences of Colorado snakes for arena zones containing house mouse odors were clear and unequivocal, but significance was detected in only one of several dependent measures among the Wyoming snakes exposed to deer mouse chemicals. The tongue–VNO system may have played a role in this effect. Although we do not view the latter as strongly supportive of a major role for deer mouse odors in signaling Wyoming snakes as to the presence or absence of prey in a particular area, those from northeastern Colorado probably do employ rodent chemicals to select foraging or ambushing sites. Field studies of foraging behavior and ecology of *C. v. viridis* occurring in certain portions of northeastern Colorado are needed.

Why are there differences in response to prey chemicals between *C. v. viridis* from northeastern Colorado and those from southcentral Wyoming? Several possible explanations exist. First, the animals studied in Colorado were long-term captives that were maintained on and tested with house mice. Conversely, the Wyoming snakes had just emerged from hibernation and were recently captured prior to experimentation, and they were tested with chemicals from a natural prey type. It is not clear how these factors may have given rise to the disparate results reported here. Second, deer mice on the Wyoming site are true desert rodents, in that they drink little water and urinate rarely, hence the two-week period to develop odorous deer mouse cages employed in the Wyoming experiments. House mice, on the other hand, urinate copiously and may be significantly more odorous than deer mice. Perhaps soiled house mouse cages represented a more potent chemical stimulus. Third, certain procedures differed between the two sets of experiments. For example, test arenas in Colorado were considerably larger than those used in Wyoming. Again it is not clear how this difference may have affected results, but it must be considered. And fourth, the Colorado snakes were maintained on one mouse every two weeks, while the Wyoming animals had not eaten for at least eight months prior to testing.

Another, perhaps more fundamental, factor is the different habitat and community selection regimes the two populations of snakes have encountered in their recent evolutionary histories. As noted above, it is possible that *C. v. viridis* from northeastern Colorado and southcentral Wyoming may be sit-and-

wait versus active foragers to varying extents. Based upon what we know about the two regions, we would predict that the Colorado animals would tend towards the "ambusher" end of a conceptual foraging continuum, with Wyoming snakes tending more towards the other "active, opportunistic forager" end. This is because simulation models indicate that a pure sit-and-wait ambushing foraging strategy among rattlesnakes is viable only when prey units are uniformly distributed and relatively close together (Duvall et al., unpublished data).

Rattlesnakes in Wyoming continue to move and search in the spring until rodents actually are encountered or captured (King and Duvall, 1990). Deer mouse exudates may not provide migrating snakes with sufficient information to make accurate decisions about the likelihood that these rodents are or are not present in an area. Perhaps the situation is different in northeastern Colorado, allowing snakes to select ambush sites that provide a good probability for success, based upon presence of prey chemicals alone. In this context, it is interesting that the preference Colorado snakes exhibited for soiled house mouse cages during days 15–17 disappeared by days 18–20. Although this may have been due to volatilization of important chemicals, the failure to actually capture prey might also have played a role. Perhaps the actual capture of prey determines the choice of foraging or ambushing sites.

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Letter to the Editor

VOMEROLFACTION AND VOMODOR

The major chemical senses of vertebrates are gustation, olfaction, and the sense supported by the vomeronasal system. The vomeronasal system is widespread in the tetrapods, being absent or vestigial only in crocodilians, some arboreal lizards, some aquatic turtles, birds, and adults of aquatic mammals and higher primates (Bertmar, 1981). Oddly, there is no term to denote chemical perception by the vomeronasal sense. At present, one must resort to vomeronasal sense, vomeronasal olfaction, or similar phrases. To distinguish it from the vomeronasal sense, olfaction is sometimes called nasal olfaction. Such terms do not pose a great problem for occasional use, but with the steadily increasing volume of papers on the structure, function, and behavioral roles of the vomeronasal system, especially in mammals and squamate reptiles (see Duvall et al., 1986), a term distinguishing the chemical sense supported by the vomeronasal system from that supported by the olfactory and gustatory systems is needed. Similarly, a term equivalent to odor for the stimuli sensed by the vomeronasal system would be useful.

The vomeronasal sense is similar to olfaction, but its anatomical substrates and sensitivities are distinct (Parsons, 1970, Halpern, 1983). Briefly, the vomeronasal organ has a sensory epithelium that is histologically quite similar to the olfactory epithelium, although there are differences in numbers of bipolar cells, cellular structure (including lack of cilia in bipolar neurons of the vomeronasal epithelium), and binding of cells to monoclonal antibodies against lactoseries carbohydrates (Halpern, 1983; Wang and Halpern, 1980; Mori, 1987). The neural connections also differ. Whereas the olfactory nerves terminate in the primary olfactory bulbs, the vomeronasal nerves terminate in the accessory olfactory bulbs. A further distinction is that the vomeronasal and olfactory systems are most sensitive to substances having different molecular weights and appear to have different behavioral roles (e.g., Cowles and Phelan, 1958; Stoddart, 1980). At least in squamate reptiles, the vomeronasal system can detect volatile substances (Burghardt, 1980), but unlike the primary olfactory organ, it is quite sensitive to compounds of very high molecular weight (Stoddart, 1980, Burghardt et al., 1988). In squamate reptiles, the vomeronasal system detects molecules of high molecular weight, often sampled from substrates; the olfactory system detects mainly volatile airborne substances (Halpern, 1983).

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Stimuli for the olfactory system are called odors and are usually restricted, in terrestrial animals, to volatile airborne substances (e.g., Geldard, 1972). Olfactory detection of volatiles by squamate reptiles may lead to activation of tongue-flicking to sample compounds of higher molecular weight that may contain greater information content useful in specific identification of the source (Cowles and Phelan, 1958; Duvall, 1981). Thus, the vomeronasal sense is important in precise identification of potential mates and their reproductive condition, potential sexual competitors, kin, prey, and important predators (numerous papers in and cited in Duvall et al., 1986, early papers reviewed by Burghardt, 1970).

It is therefore useful to make a distinction between primary olfaction and vomeronasal olfaction, ideally by a one-word descriptor equivalent to gustation and olfaction. Such a term should readily identify the sensory system in question, as does the simple description of animals unable to detect chemicals by the vomeronasal systems as avomic (Graves and Duvall, 1985). After considering various alternatives, we propose the term vomerolfaction. This term emphasizes the distinctness from olfaction, yet gives recognition to the similarity. To denote the largely nonvolatile chemical stimuli sensed by vomerolfaction, we propose the term vomodor.

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INTRODUCTION TO BACTERIAL MOTILITY AND CHEMOTAXIS

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Abstract—Bacteria swim by rotating semirigid, left-handed helical flagellar filaments; counterclockwise (CCW) rotation produces straight swims, known as "runs," and clockwise (CW) rotation generates abrupt changes in direction, known as "tumbles." As a cell moves through its environment, alternately running and tumbling, it detects spatial gradients of attractants and repellents by making temporal comparisons of their concentration. These chemicals bind to receptors in the cell envelope to modulate the activity of the chemotactic signal transducers, proteins that span the cytoplasmic membrane. Signals generated by the transducers control the motion of the flagella to promote migration up attractant gradients and down repellent gradients. Chemotactic adaptation, accomplished by methylation–demethylation of the transducers, cancels out these signals. Adaptation is an essential component of the "memory" that allows bacteria to use a temporal mechanism to detect spatial gradients. Both signaling and adaptation are mediated by changes in the level of phosphorylation of several cytoplasmic chemotaxis (Che) proteins. The activity of the transducers regulates the rate of autophosphorylation of the CheA protein, which then passes the phosphate on to other proteins. In particular, phosphorylated CheY protein controls the frequency of tumbling because it promotes CW flagellar rotation, and the CheB esterase modulates adaptation because its nonphosphorylated form removes methyl groups from the transducers much more slowly than its phosphorylated form.

Key Words—Flagella, motility, chemical gradients, temporal sensing, bacterial memory, chemoreceptors, signal generation, intracellular signaling, protein phosphorylation, protein methylation, adaptation, Bacterium.

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INTRODUCTION

The intent of this article is to provide the nonspecialist with a brief overview of bacterial motility and chemotaxis. The acronyms for the various genes and proteins will be explained as they appear in the text. This field has been the subject of several recent and excellent reviews, to which I direct readers interested in a more detailed treatment (Macnab, 1987a,b; Stewart and Dahlquist, 1987; Adler, 1986; Ordal, 1985; Hazelbauer and Harayama, 1983; Koshland, 1980).

FLAGELLAR ROTATION AND THREE-DIMENSIONAL RANDOM WALK

Bacteria swim by rotating semirigid, helical flagellar filaments. This rotation is driven by a reversible electrostatic motor located at the base of each flagellum. The motors rotate in the plane of the cell envelope; the rotation is bent 90° around the body of the cell through a flexible coupling called the proximal hook. The hook connects the filament to the basal body of the flagellum.

In most bacteria, rotation is powered by an inward current of H^+ ions, which presumably passes through or between flagellar components associated with the cytoplasmic membrane. In some alkalophilic species, rotation is driven by an inward Na^+ current. Under contrived, nonphysiological circumstances, an outward flux of H^+ also causes flagella to turn.

In the enteric bacteria—the exclusive subject of the remainder of this essay—and in many other bacterial groups as well, flagella appear to arise at random sites on the surface of the cell. Evidence indicates that the chemoreceptors of these bacteria are also randomly distributed on the cytoplasmic membrane and are not associated with the flagella in any ordered way. This spatial separation of receptors and flagella requires that the two elements be coupled by a transduction mechanism that transmits information over the dimensions of a bacterial cell (several micrometers).

In the absence of environmental input, cells of *Escherichia coli* move in a three-dimensional random walk. This walk consists of alternating "runs" (periods of one to several seconds in which the cell swims in a continuous path) punctuated by "tumbles" (periods on the order of 0.1 sec when the cell actively, but randomly, changes its heading). During a run, counterclockwise (CCW) rotation of the flagellar motor causes the six to eight left-handed-helical flagellar filaments to coalesce into a bundle that pushes the cell along its path. During a tumble, clockwise (CW) rotation of the flagella causes the bundle to fly apart, leading to rapid reorientation of the cell. Resumption of CCW rotation allows the bundle to reform, and another run ensues.

E. coli cells respond to spatial gradients of attractant by extending the length of a run in the favorable direction—up an attractant gradient or down a repellent gradient. Thus, the primary response to an attractant is suppression of the CW flagellar rotation that generates tumbling, and chemotaxis is effected by altering the steady-state CCW to CW switching probability of the flagellar motor.

SPATIAL GRADIENTS SENSED BY TEMPORAL MECHANISM

Spatial gradients are sensed by comparing chemoeffector concentrations in time as the cell swims. (See the discussion by H.C. Berg, p. 119 in this issue). In effect, cells are able to compare the instantaneous chemoeffector concentration, measured by receptor occupancy, with the concentration over the last few seconds.

In receptors utilizing methyl-accepting chemotaxis proteins (MCPs), more commonly referred to as signal transducers, this "memory" component is the extent of carboxyl methylation of specfic glutamate residues in the cytoplasmic portion of the transmembrane transducer. Occupancy by attractants leads to increased methylation and occupancy by repellents to demethylation relative to the unstimulated level.

Since the methylation reaction is slow relative to ligand binding and dissociation, the degree of methylation reflects the receptor occupancy of the recent past. A transducer that is undermethylated with respect to its ligand occupancy signals the flagella to turn CCW. Conversely, a transducer that is overmethylated with respect to its ligand occupancy signals the flagella to turn CW. A transducer that is in balance generates a level of signal that leads to switching between the CCW and CW states. The level of methylation that creates a balanced situation is low, medium, and high for a repellent-saturated, unstimulated, and attractant-saturated MCP, respectively.

MOLECULAR NATURE OF CHEMORECEPTORS

Bacterial chemoreceptors can be of several types. The periplasmic portions of several of the transducers discussed in the previous section serve as primary receptors. Thus, the Tar (taxis toward aspartate and away from some repellents) protein binds aspartate directly, and the Tsr (taxis toward serine and away from some repellents) protein binds serine. Both of these amino acids are potent attractants for *E. coli*. They may interact with a positively charged "arginine pocket" in their respective transducers (Wolff and Parkinson, 1988; Dahl and Manson, 1989).

Other attractants first interact with substrate-binding proteins, which are

localized in the periplasmic space. The binding of ligand induces a conformational change in these proteins that increases their affinity for a specific signal transducer: Trg (taxis toward ribose and galactose) for the ribose- and galactose-binding proteins, Tap (taxis toward peptides) for the dipeptide-binding protein, and Tar for the maltose-binding protein. The latter transducer is unique in that it has both an amino acid (aspartate) and a protein as excitatory effectors.

Receptors for other attractants are less well characterized. Many sugars are sensed chemotactically by the phosphotransferase system (PTS). Unlike the transducer-mediated responses described above, however, the transport and concomitant phosphorylation of the substrate is essential. The nature of the signal and of the adaptation process during PTS chemotaxis is a matter of active investigation.

Oxygen taxis, also known as aerotaxis, also requires chemical modification of the chemoeffector, since the receptor in this instance appears to be cytochrome oxidase. It is believed that the signal generated by oxygen is an increase in the protonmotive force (PMF), but the manner in which this increase is monitored by the cell, and how it is used to regulate the flagellar switch, is unknown. It has been suggested that PMF taxis, in which the energy level of the cell is sensed, could be the ancestral taxis and thus exhibits the most fundamental mechanism of flagellar control.

The methyl-accepting transducers are involved in the responses to many repellents, but the nature of the stimulation is in general poorly understood and, unlike the situation for attractants, may in some cases be indirect. Weak organic acids seem to exert their effect by permeating the cell membrane in the protonated form, thereby lowering the cytoplasmic pH and affecting the degree of protonation of a critical histidine residue in the signaling region of the transducer.

NATURE OF INTRACELLULAR SIGNAL

Recent work in several laboratories has enormously increased our understanding of the chemotactic signal in bacteria. No detailed information is available about how the receptor and signaling domains of the transducers communicate across the membrane. However, based on the analysis of mutant transducers with locked signal outputs, Ames and Parkinson (1988) have proposed that an alteration in the conformation of the receptor portion of the transducer is transmitted to the signal domain through a membrane-spanning linker region. The resulting change in the conformation of the signal domain is then canceled by increased methylation.

A series of papers from the laboratory of Melvin Simon (Hess et al., 1987;

1988a,b; Oosawa et al., 1988) has demonstrated that the communication between the signaling domain of the transducers and the flagellar switch involves a phosphorylation cascade. The CheA protein (one of six soluble, cytoplasmic proteins required for any chemotaxis) autophosphorylates, using ATP as the phosphate donor. This phosphate is linked to a histidine residue in the N-terminal portion of the protein. The phosphate group can be transferred to the CheY protein. Phosphorylated CheY, in turn, interacts with the switch at the base of the flagellum to initiate CW rotation. The transducers, with the participation of the CheW protein, modulate the rate of CheA autophosphorylation. Mutants lacking any one of the CheA, CheW, or CheY proteins, or all of the major transducers, can rotate their flagella only in the CCW direction, and thus are "smooth swimmers."

The CheZ, CheR, and CheB proteins also play essential roles in signaling and adaptation. CheZ antagonizes the effect of CheY by accelerating the dephosphorylation of CheY, thereby canceling CW input to the flagellar switch. CheZ may also interact directly with the switch to promote CCW rotation, although this interaction has not been established unequivocally. A mutant defective in CheZ function has increased CW rotation of its flagella, and the mutant cells tumble incessantly.

Mutants lacking CheR, the transferase enzyme that adds methyl groups to specific glutamate residues in the cytoplasmic domain of the transducers, are defective in adaptation. Normally, the undermethylated transducers send out a CCW signal, and CheR mutants swim smoothly, but a repellent stimulus initiates an abnormally extended period of tumbling because of nonadapting CW rotation of the flagella. The methyltransferase uses S-adenosylmethionine as a methyl-group donor and seems to operate at a constitutive rate.

Changes in the net level of transducer methylation are generated by controling the activity of the CheB esterase, which removes methyl groups from the glutamyl-methyl esters. The N-terminal region of CheB is a another substrate for phosphorylation by CheA. CheB-phosphate has greatly enhanced esterase activity, which is catalyzed by the C-terminal portion of the protein.

Upon binding an attractant or ligand-associated binding protein, a conformational change initiated in the periplasmic domain of the transducers is propagated through the membrane to the cytoplasmic signaling domain. This attractant-activated state of the signaling domain, perhaps acting together with the CheW protein, inhibits the basal rate of autophosphorylation of CheA that is maintained by the transducers in the absence of external stimuli. The level of CheY phosphate falls rapidly because of the presence of CheZ protein, and the flagellar motors spin CCW. At the same time, however, the level of CheB phosphate also falls, and demethylation of the transducers is reduced. As a consequence, the extent of transducer methylation increases, which in turn

blocks the signal from the transducers that inhibits autophophorylation of Che A. The cell adapts, and its flagella return to the unstimulated pattern of alternating CCW-CW rotation.

Repellents presumably elicit a conformational change in the transducer that stimulates autophosphorylation of CheA, again perhaps with the mediation of CheW. The level of CheY phosphate increases rapidly, and CW rotation of the flagella ensues. At the same time the amount of CheB phosphate also is increased, leading to rapid removal of methyl groups from the transducer, which negates the stimulation of CheA autophosphorylation. Once again, the cell adapts, and the normal pattern of alternating CCW-CW rotation is restored. In general, cells respond to the removal of an attractant as they do to the addition of a repellent and to the removal of a repellent as they would to the addition of an attractant.

Not all aspects of this model have been verified by experiment. However, the scheme provides a good working hypothesis for directing future experiments.

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The following are abstracts from the Symposium on Sensory Biology of Microorganisms and although thematically related, are not part of the preceding article.

METHYL-ACCEPTING CHEMOTAXIS PROTEINS: TRANSMEMBRANE RECEPTORS IN BACTERIA

Gerald L. Hazelbauer, Biochemistry/Biophysics Program, Washington State University, Pullman, Washington 99164. Methyl-accepting chemotaxis proteins are central components of the extensively studied chemotactic system of the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*. These transmembrane receptor proteins, called transducers, consist of an amino-terminal, extracytoplasmic, ligand-recognition domain and a carboxy-terminal, cytoplasmic, signaling, and adaptation domain, connected by two transmembrane sequences. Adaptation is linked to methylation of specific glutamyl residues in the cytoplasmic domain. The four transducers in *E. coli* exhibit extensive sequence identity in the cytoplasmic domain, consistent with the common functions in sensory signaling and adaptation. Methyl-accepting chemotaxis proteins have been observed in a number of other bacterial species, and this laboratory became interested in their relationship to the transducer proteins in the enterics. Initial studies with antiserum that recognized transducers from *E. coli* indicated that methyl-accepting proteins from *Bacillus subtilis* and *Spirochaeta aurantia* were antigenically related to the enteric proteins. More recent immunoblot studies have identified candidate transducers in a number of eubacterial species. These observations prompted a detailed investigation of methyl-accepting taxis proteins in an archaebacterial species, *Halobacter halobium*. The results revealed that the basic themes observed in the study of methyl-accepting chemotaxis proteins from the enterics persist in this representative of the archaebacteria, although some interesting variations occur.

In collaboration with a number of other research groups, we are currently searching in a number of bacterial species for genes that code for methyl-accepting sensory proteins. A series of oligonucleotide probes, representing regions of conserved sequence among the transducer genes of *E. coli*, are being used to search for complementary sequences in chromosomal DNA or cloned frag-

ments. Promising candidate restriction fragments have been detected in *H. halobium*, *Rhizobium meliloti*, and *Pseudomonas putida*. Cloning and sequencing of the genes contained on these fragments should provide insight into the degree to which the structure of these sensory proteins has been conserved over the evolutionary distances that separate the diversity of bacterial species.

MOTILITY AND CHEMOTAXIS OF AN AQUATIC SPIROCHETE

E.P. Greenberg, Department of Microbiology, University of Iowa, Iowa City, Iowa 52242. *Spirochaeta aurantia* serves as the model for studies of spirochete chemotaxis. The natural habitat of *S. aurantia* is anoxic muds at the bottom of freshwater ponds. Like all spirochetes, *S. aurantia* is a slender helical bacterium possessing flagella enclosed entirely within the periplasmic space. In the case of *S. aurantia*, the flagella operate by working against components of the cell rather than working against the external environment as do the flagella of bacteria other than spirochetes. The unusual motility system of spirochetes allows these organisms to move through viscous gels that immobilize most other bacteria; this is thought to be important to the ecology of these organisms. Attractants for *S. aurantia* are sugars, while primary repellents include acids and alcohols. Because of the unique motility of spirochetes, it has been of interest to compare the mechanism of chemotaxis in *S. aurantia* to the well-characterized mechanism of chemotaxis in *E. coli*. Although many of the components of the motility and chemotaxis machinery are analogous in the two organisms, there appear to be some very interesting differences. In particular, the signals that coordinate motility are postulated to be different. Studies involving automated motion analysis and chemotaxis mutants will be particularly stressed during the talk.

THE ROLE OF BACTERIAL CHEMOTAXIS IN INITIATION OF *Rhizobium*/LEGUME SYMBIOSIS FOR NITROGEN FIXATION

Kostia Bergman, Department of Biology, Northeastern University, Boston, Massachusetts 02115. Many types of bacteria are equipped for chemotaxis, the movement toward or away from specific chemicals. Investigators of this behavior in enteric bacteria have often emphasized its importance as a model for sensory physiology, or even as a model for the central nervous system of higher organisms. Our long-term aim is to extend the powerful methods developed in such work toward an understanding of the role that bacterial chemotaxis has in microbial ecology.

It is now clear that initiation of the *Rhizobium*/legume symbiosis for nitrogen fixation involves the exchange of multiple signals between the host plant

and the microsymbiont. We have demonstrated that motile rhizobia accumulate at localized sites of attractant release on the surface of the plant root. The localized sites are found in the area of the root shown by others to be susceptible to the infection process which initiates symbiotic nitrogen fixation.

Studies with behavioral mutants of the rhizobia demonstrate that this response requires a functional system for the sensory and motor processes of chemotaxis. Furthermore, competition experiments with strains that are isogenic except for known mutations in their chemotaxis system suggest that this behavior has a role in the physiology of the interaction between the two organisms, presumably guiding the bacteria to potential infection sites.

We have used a soft-agar swarm-plate assay to test the attractant activity of purified chemicals and plant root extracts. This assay is made specific by the use of two mutants of *Rhizobium meliloti* strain SU47 that no longer detect any pure substance tested (primarily sugars and amino acids) but still detect the localized-site attractant. Partial purification of active substances has shown that they are related to amino acids and are distinct from the known *nod* gene activators found by other investigators. However, recently Caetano-Anolles et al. (*J. Bacteriol.* 170:3164–3169, 1988) have shown, using capillary assays, that at low concentrations luteolin, a *nod* gene activator, is also an attractant. The possible advantages of such a two-attractant system for fine-tuning the response will be discussed.

AUTOINDUCTION OF BACTERIAL BIOLUMINESCENCE: A MECHANISM FOR ENVIRONMENTAL SENSING

Kenneth H. Nealson[1] *and Reinhardt A. Ronson, Center for Great Lakes Studies, University of Wisconsin–Milwaukee, Milwaukee, Wisconsin 53204.*
Autoinduction is the process that controls enzyme synthesis, and thus total bioluminescence, in marine luminous bacteria. As the bacteria grow, they continuously produce a small autoinducer (AI) molecule, which accumulates in the medium. When AI reaches a critical concentration, it acts as the specific inducer for the bioluminescence (*lux*) system; luminescence is induced by a factor of 100 or more. The autoinduction phenomenon is outwardly similar for several species of marine bacteria but has been carefully characterized only for *Vibrio fischeri*. The AI for *V. fischeri*, [*N*-(3-oxohexanoyl)-3-aminodihydro-2[^{3}H]-furanone] has been isolated, identified, and synthesized. The addition of synthetic AI results in the rapid induction of the *lux* system in mutants lacking AI. Genetic experments have shown that the *lux* regulon from *V. fischeri* consists of seven genes, two of which are regulatory. One of these codes for a protein responsible

[1] Symposium participant.

for AI production, and the other codes for a protein necessary for AI to exert its effect. Taken together, these results can be assembled into a model that predicts that cells maintained at low density will be continuously dark, while those present at higher densities will be subject to autoinduction, and thus be bright. Experiments in chemostats have shown that cells maintained at low density remain dark unless AI is supplied in the medium, indicating that the induction mechanism involves an extracellular phase. This result supports the view that autoinduction acts as a mechanism for environmental sensing.

CELL-CELL INTERACTIONS IN *Myxococcus*

Dale Kaiser,[1] *Lee Kroos, Adam Kuspa, Yvonne Cheng, and Seung Kim, Department of Biochemistry, Stanford University, Stanford, California 94305.* When starved, 10^5 *Myxococcus* cells congregate to form a fruiting body within which some cells become spores. Many new proteins appear during fruiting body development. A transposable promoter probe, Tn*5 lac*, can express beta-galactosidase (BG) if it inserts with its left end downstream of an active promoter. Among 2374 independent insertions of Tn*5 lac* into *Myxococcus*, 37 were identfied that make at least three times more BG after fruiting body development starts than during growth. Each of these transcriptional fusion strains increases BG at a particular time in development, ranging from before aggregation to the time of sporulation. This set of fusion strains serves as an indicator of progress through the developmental program.

How is the time of morphological, biosynthetic, and gene expression events controlled? A set of mutants has been isolated that behave as if they are defective in cellular interactions necessary to development. Sporulation in fruiting bodies is restored to the mutants if they are mixed with developing wild-type cells. "Complementation" experiments with whole cells divide the mutants into four groups (A, B, C, and D). Particular sets of *lac* fusions depend on the normal function of complementation groups. For example, A^- strains containing 18 of the 21 different *lac* fusions tested fail to make BG, while the other three strains continue to make BG. The pattern of dependence of BG expression on the A, B, C, and D complementation groups is consistent with a branched-linear regulatory pathway (L. Kroos and D. Kaiser, *Genes Dev.* 1:840–854, 1987).

A-dependent and C-dependent *lac* fusions are being used to purify the molecules responsible for an early (A) and late (C) developmental signal. In crude extracts of developing wild-type cells, both activities are heat labile and nondialyzable (A. Kuspa et al., *Dev. Biol.* 117:267–276, 1986).

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ION CHANNELS IN *Paramecium* YEAST, AND *Escherichia coli*

Ching Kung, Laboratory of Molecular Biology and Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706. Animal behavior is largely governed by biological electricity. Ions, the carriers of electric currents, cannot go through the hydrophobic lipid bilayer of the membrane. Instead, they go through a special class of integral membrane proteins called ion channels. Because open channels drain the electrochemical gradients, they are "gated" and only open upon sensing certain environmental signals. Thus, channels are switches that transduce the signals into electric or ionic information within the cells. Of the approximately 60 different ion channels now recognized, some are gated by external ligands, internal messengers, transmembrane voltages, or mechanical forces. Although ion channels have traditionally been studied in higher animals, we have chosen to study the channels in microbes.

Paramecium, the ciliated protozoan, is a giant among microbes and has been investigated with conventional electrophysiological procedures. At least eight electric currents, passing through eight distinct ion channels, can be recognized. Mutants defective in individual currents have been isolated and studied. For example, mutations in seven genes each result in a decrease or the disappearance of Ca^{2+} current. In the case of a group of mutants called *pantophobiacs*, which lack Ca-gated K^+ currents, the molecular defect has now been traced to calmodulin (Schaeffer et al., *Proc. Natl. Acad. Sci. U.S.A.* 84:3931–3935, 1987). *Pantophobiac A*, for example, was found to have a serine-to-phenylalanine substitution at residue 101 of calmodulin.

Smaller microbes now can be studied electrically using the patch-clamp technique. The budding yeast *Saccharomyces cerevisiae* has the ideal size for patch-clamp experiments. Removal of the cell wall with zymolase yields spheroplasts of 5–7 μm in diameter. We found two types of ion-channel activities in yeast. One is a voltage-gated K channel, which has a unitary conductance of 20 picoSiemens (pS). It opens when the membrane depolarizes and passes K^+ specifically. Based on gating mechanism, conductance, ion selectivity, and blocker spectrum, this channel is very similar to the voltage-gated K^+ channels that turn off the action potential in nerves and muscles. The second type of channel we found in yeast is gated by mechanical forces instead of voltages. These channels tend to open when a small suction is applied to the membrane patch. This type of channel has little ion selectivity.

Normal *E. coli* cells are too small even for the patch clamp. However, giant spheroplasts, 5–10 μM in diameter, can be generated for such studies. We found a voltage-gated channel that opens more frequently upon membrane depolarization. It has a unitary conductance of 450 pS and appears to have no ion selectivity. The second channel we encountered is gated both by voltage

and suction. It has a large unit conductance of nearly 1000 pS. It passes inorganic ions as well as organic ions as large as glutamate.

These are the first patch-clamp studies on yeast and *E. coli*. Although the physiological roles of ion channels in these microbes are not yet known, we hope that the combination of biophysical studies and the well-advanced genetics and molecular biology for these organisms will lead to a deeper understanding of the structures, functions, and evolutionary origins of ion channels.

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PHYSICAL CONSTRAINTS ON MICROBIAL BEHAVIOR
How You Act if You Are Very Small

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Abstract—Physical constraints limit the way in which an organism as small as *Escherichia coli* can interact with its surroundings. These constraints are listed, together with references to the relevant literature.

Key Words—Chemotaxis, sensory transduction, diffusion, viscous flow, *Escherichia coli*, Bacterium.

The bacterium *Escherichia coli* is remarkably small, some 10^{-4} cm in diameter. It swims by rotating about six thin helical filaments that arise at random points on its surface. The motor that drives each filament is embedded in the cell wall and the cytoplasmic membrane and is smaller in diameter than the cell by a factor of about 50. In the absence of a stimulus, *E. coli* swims steadily at speeds on the order of 20 diameters per second. It executes a random walk with runs (intervals during which it swims along a gently curved path) of about 1 sec duration and tumbles (intervals during which it moves erratically with little net displacement) of about 0.1 sec duration. During runs, the filaments turn counterclockwise and work coherently in a bundle that pushes the cell forward. During tumbles the filaments turn clockwise and work independently, moving the cell this way and that. When the cell swims in a spatial gradient of a chemical attractant, runs that happen to carry it up the gradient are extended, while those that happen to carry it down the gradient are not. Thus, the cell drifts in a favorable direction by executing a biased random walk. The bias is positive, not negative: the runs get longer, not shorter.

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Physical constraints influence this sensory strategy in several ways—I quote from a recent survey (Berg, 1988): (1) The flagellar filaments are long, thin, and helical, because motion is dominated by viscous rather than inertial forces: it sounds contradictory, but thrust must be generated by viscous drag. (2) A cell is unable to run in a straight line, because rotational perturbations due to Brownian movement knock it off its path: *E. coli* forgets where it is going in about 10 sec. (3) A cell cannot improve its lot locally by swimming or stirring, because transport of small molecules is effected by diffusion rather than bulk flow: local displacements of the fluid do not increase the cells nutritional intake. (4) A cell must sense a chemical gradient temporally rather than spatially, because comparisons between concentrations in front or behind are overwhelmed by diffusive currents due to its rapid motion. Although the net increase in intake is very small, a cell always absorbs more molecules in front than behind. Therefore, with a spatial mechanism, any new direction is deemed favorable. Finally, (5) the precision with which a cell can make temporal comparisons is limited by statistical fluctuations. The counting statistics improve with the square root of the product of the concentration and the integration time. Therefore, a chemical cannot be sensed at an arbitrarily low concentration, because the integration time required would be prohibitively long.

For a brief review of bacterial chemotaxis with an emphasis on the physiology, see Berg (1988). For a taste of the physics in a back-of-the-envelope style, see Purcell (1977) and Berg (1985). For a more extensive textbook discussion, see Berg (1983). For a rigorous treatment of the counting problem, see Berg and Purcell (1977).

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PHEROMONE INTERACTIONS AND IONIC COMMUNICATION IN GAMETES OF AQUATIC FUNGUS *Allomyces macrogynus*

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Abstract—The flagellate male and female gametes of the aquatic fungus *Allomyces macrogynus* are each attracted to a sexual pheromone produced by the opposite gamete type. The sperm attractant, sirenin, causes chemotaxis to female gametes. Examination of sperm chemotaxis shows that the pheromone influences the frequency of directional changes and the duration of a chemotactic run. Physiological experiments using tertiary amine local anesthetics or calcium chelators such as EGTA demonstrate that sirenin stimulates the influx of calcium ions (Ca^{2+}) into the sperm cytoplasm. Radiological experiments with $^{45}CaCl_2$ have demonstrated this calcium flux directly. Structurally, sirenin is an oxygenated sesquiterpene that consists of a cyclopropyl ring attached onto an isohexenyl side chain. The pheromone displays a threshold concentration for attraction at 10 pM in chemotaxis bioassays. Structure-activity relationships with racemic sirenin and sirenin analogs indicate that biological activity requires a terminal hydroxymethyl group on the side chain. In addition, a hydrophobic group must be present at the other end of the sirenin molecule. Besides sirenin, the sperm cells of *A. macrogynus* produce a female attractant, parisin. While the molecular nature of this attractant is not completely resolved, some general features of the molecule suggest it may be similar structurally to sirenin.

Key Words—*Allomyces macrogynus*, sex pheromones, sesquiterpene, sirenin, parisin, analogs, calcium ions, chemotaxis, fungus.

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INTRODUCTION

Overview of the Organism. Chemical communication is an important process in both prokaryotic and unicellular eukaryotic organisms (Taylor and Panasenko, 1984). In the fungi and algae, chemical communication between motile gametes can involve sexual pheromones that are produced to attract mating partners. Although several algal pheromones in the division Phaeophyta have been described (see Maier and Müller, 1986), few sexual pheromones have been discovered in the aquatic fungi (division Chytridiomycota). This paper discusses the sexual pheromones of the aquatic fungus *Allomyces macrogynus* and describes what is known about the chemical composition of these pheromones. For the sperm-attractant sirenin, its possible mode of action will be discussed.

The genus *Allomyces* (class Chytridiomycetes) is a subtropical water mold that grows as a saprophyte on decaying plant and animal matter (Emerson, 1941). In the sexual phase of *A. macrogynus*, multinucleate male and female reproductive structures, called gametangia, are formed at the tips of the growing vegetative hyphae. The two types of gametangia are easily discerned with a dissection or compound microscope. The male gametangia are terminal, orange, and smaller than the subterminal, colorless, female gametangia. Under conditions of nutrient starvation, each female and male gametangium undergoes cytoplasmic cleavage (Sewall and Pommerville, 1987) and differentiates approximately 50 female gametes and 100 sperm. Both types of gametes are uninucleate and swim using a posterior whiplash flagellum (Pommerville and Fuller, 1976). These motile cells also are easily identified with a compound microscope. The sperm cells are orange, highly motile, and about half the size (5 μm) of the colorless, sluggishly motile female gametes. Cell communication is exhibited only during this motile stage in the life cycle. Communication is important for efficient gamete mating and zygote formation (Pommerville, 1982; Pommerville and Fuller, 1976).

Overview of Chemotaxis. The sperm and female gametes of *A. macrogynus* each generate a specific pheromone to attract the opposite gamete type (Pommerville and Olson, 1987a). The effects of the female attractant, sirenin, on the sperm have been intensively studied, and the findings show that the sperm require a sensory system coupled to transduction processes (Carlile and Machlis, 1965a,b; Machlis, 1973; Pommerville, 1977, 1981).

Based on quantitative chemotaxis assays, Machlis (1973) suggested that sperm attraction required a pheromone gradient. However, the nature of the attraction process become clearer when sperm motility was studied by dark-field microscopy (Pommerville, 1978). In brief, sperm motility without sirenin is characterized by short, bending runs. Each run is interrupted by a brief period (<1 sec) when the sperm cell temporarily stops swimming and the cell body

pitches an average angular distance of 60°. Another run then occurs but in the direction determined by the pitch of the cell body (Pommerville, 1978).

Sperm undergoing chemotaxis in a gradient of sirenin (toward female gametes) exhibit longer runs and fewer pitches of the cell body. Furthermore, the addition of sperm to a uniform distribution of attractant results in continuous pitches of the cell body. These findings demonstrate that sirenin influences the frequency of directional changes (chemoklinokinesis) and duration of runs.

In the majority of eukaryotic microbes that exhibit chemotactic or phototactic behavior, the cells can undergo at least a temporary reversal of flagellar beat (backwards swimming). This usually results in negative chemotaxis when sensing potentially harmful compounds. The reversal of flagellar beat is due to the influx of Ca^{2+} (Holwill, 1980; Gibbons, 1982). With *Allomyces*, experimental analysis has shown that sirenin stimulates the influx of calcium ions ($[Ca^{2+}]$) into the sperm cytoplasm (Pommerville, 1981). This finding indicates that pitches of the cell body are due to increased levels of cytosolic Ca^{2+}. Removal of extracellular Ca^{2+} with EGTA results in circular swimming even when sirenin is present (Pommerville, 1981). Thus, the equivalent of flagellar reversal in sperm of *Allomyces* is pitching of the cell body. Once excess Ca^{2+} is sequestered or pumped out of the cell, another run can occur. Thus, the motile cells of *A. macrogynus* can undergo negative chemotaxis (Pommerville and Olson, 1987b), but this requires the cells to undergo several pitches of the cell body to turn around.

When sperm are placed in a solution containing tertiary amine local anesthetics (procaine or tetracaine), the sperm swim in circles without pitches of the cell body (Pommerville, 1981). The addition of sirenin to such a solution of sperm does not change the behavior of the cells. Removal of the anesthetic leads to recovery of normal motility and gamete attraction. These results can be understood based on known actions of procaine and tetracaine. These compounds displace Ca^{2+} from membranes (Low et al., 1979), interfere with normal membrane electrical properties, and act as antagonists to the normal functioning of calcium-binding proteins, such as calmodulin (Volpi et al., 1981). In summary, physiological and pharmacological experiments with chelators and anesthetics suggest indirectly that Ca^{2+} is important to the regulation of sperm chemotaxis in *A. macrogynus*.

Experiments with $^{45}CaCl_2$ have been used to demonstrate directly the influx of Ca^{2+} in *A. macrogynus*. The addition of sperm to a $^{45}CaCl_2$ solution results in the binding of $^{45}Ca^{2+}$ to the exterior of the plasma membrane (Figure 1). After washing these cells in distilled water or initially using lower concentrations of $^{45}CaCl_2$, the levels of radioactivity are reduced (Figure 1b). However, when sirenin is added to a suspension of sperm in a $^{45}CaCl_2$ solution, there is a 2.5-fold increase in radioactivity above that detected before sirenin was added (Figure 1a). The simultaneous addition of sirenin and $^{45}CaCl_2$ to a suspension

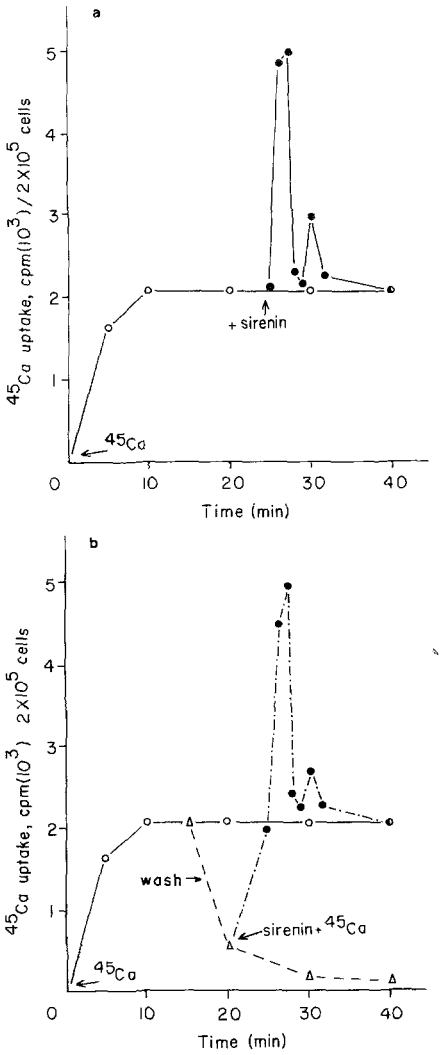

FIG. 1. Measurement of ^{45}Ca activity with and without sirenin addition. (a) Addition of sirenin to sperm incubated in ^{45}Ca resulted in a 2.5-fold increase in cell radioactivity. For these experiments, sperm in Eppendorf tubes were mixed with $^{45}CaCl_2$ in distilled water (○ —— ○) to give 2×10^5 sperm/0.4 ml (5 μM $CaCl_2$). The tubes were incubated for 24 min, at which time sirenin (● —— ●) or distilled water (○ —— ○) was added. At 1-min intervals, the solution from one tube was removed and layered over 12.5% sucrose, centrifuged for 30 sec, and the tube frozen. After a complete set of experiments, the tips of the tubes were cut off and the cell pellets placed in scintillation fluid. (b) Simultaneous addition of sirenin and ^{45}Ca to sperm that had been incubated in ^{45}Ca (○ —— ○) and washed with distilled water (△ -- △) before sirenin/^{45}Ca addition (● - · - ●). Note that addition of sirenin and $^{45}CaCl_2$ to washed cells still stimulated the same level of ^{45}Ca uptake. Radioactivity was determined as described in (a).

of washed gametes also results in a rapid influx of $^{45}Ca^{2+}$ (Figure 1b). When zoospores are substituted for sperm in these experiments, influx of $^{45}Ca^{2+}$ above membrane-bound levels is not detected.

SIRENIN, THE SPERM ATTRACTANT

Machlis et al. (1966) examined the molecular nature of sirenin by producing large quantities of the attractant from pure female strains of *Allomyces*. This provided sufficient material to isolate and purify the pheromone (Machlis et al., 1966), and to determine the structure of sirenin (Machlis et al., 1966, 1968; Nutting et al., 1968).

Sirenin is a sesquiterpenediol (Figure 2) of low molecular weight (236 daltons). Following the synthesis of *dl*-sirenin, a few isomers and analogs were generated (Bhalerao et al., 1970). When these synthetic products were tested for their ability to attract sperm cells (Machlis, 1973), only the synthetic *l*-enantiomer of sirenin was found to be active (Figure 2). The ability to exclude inappropriate synthetic sirenin analogs (the *d*-enantiomer and the *exo*-methyl diastereomer; Figure 2) was shown by their inability to attract sperm in chemotaxis bioassays (Machlis, 1973). When *d*-sirenin was mixed with *l*-sirenin, there was no decline in or interference with chemotaxis. These findings provided the first indirect evidence for pheromone receptors in *Allomyces*.

To understand receptor recognition further, we have synthesized several

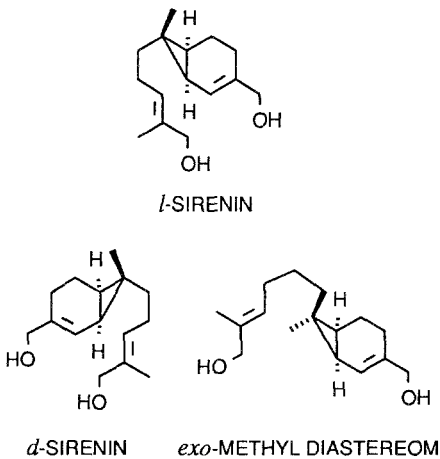

FIG. 2. Structure of *l*-sirenin and two of the analogs tested by Machlis (1973). Both the *d*-enantiomer and the *exo*-methyl diastereomer were inactive in attracting sperm in a chemotaxis bioassay.

active sirenin analogs and tested them for their ability to attract sperm in a bioassay (Pommerville, 1987; Pommerville et al., 1988). The most potent of these compounds is a monohydroxy analog (Figure 3A), which exhibits identical biological activity to the natural pheromone. Sperm respond to the monohydroxy analog and to racemic sirenin with threshold concentrations (i.e., the minimal concentration needed to stimulate male chemotaxis in a bioassay) of 10 pM. The other analogs we synthesized were either inactive or exhibited much higher threshold concentrations (10 μM or higher). However, sperm attraction at these higher analog concentrations still varied with their structural resemblance to sirenin (see Pommerville et al., 1988). Indeed, the benzyl ether of racemic sirenin (Figure 3B), which has a threshold activity of 10 μM, again supports the need for receptor recognition.

Important structure–activity relationships can be discerned by comparing the relatively inactive compounds (Figure 3B–E) with the monohydroxy analog (Figure 3A) and *l*-sirenin (Figure 2). First, the hydroxymethyl group attached on the bicyclic ring of sirenin is not necessary for activity (compare Figure 2 and Figure 3). The monohydroxy analog was as potent as sirenin. Second, replacement of the hydroxymethyl group on the ring with a large hydrophobic group (benzyl ether) reduces activity substantially (Figure 3). Thus, chemotactic activity in the 10 pM range requires a structure having a terminal hydroxy-

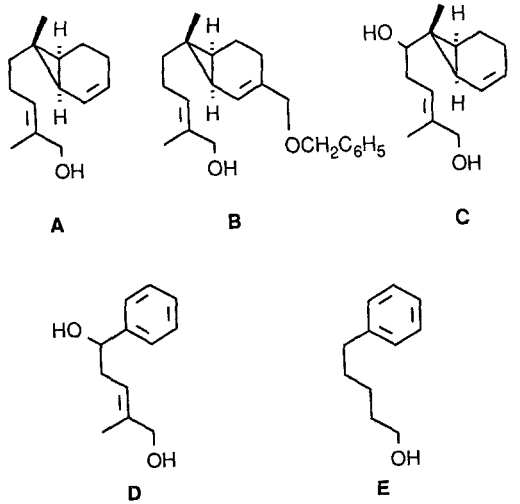

FIG. 3. Structures for several active analogs of sirenin. The monohydroxy analog (A) had a threshold concentration at 10 pM, identical to racemic sirenin. Analogs B–E all had threshold concentrations at 10 μM. See text for details. (Adapted from Pommerville et al., 1988.)

methyl group on the side chain and a large hydrophobic group at the other end of the molecule. Any structural change that leads to improper fit at the receptor's recognition site requires an attractant concentration of at least 10 μM for a chemotactic response—if indeed any response occurs (Pommerville et al., 1988).

Sirenin must bind in a conformation that distinguishes it from the *d*-enantiomer and other analogs. We postulate that when *l*-sirenin is bound, the hydroxymethyl group on the ring is located in a nonspecific hydrophilic region (Figure 4A). Thus, the monohydroxy analog is a potent attractant. *d*-Sirenin lacks activity because its hydroxymethyl group intrudes into a region of the receptor that must be occupied by the hydrophobic ring of *l*-sirenin (Figure 4b).

PARISIN, THE FEMALE ATTRACTANT

When sperm and female gametes are physically separated from one another in a bioassay, the sperm cells attract female gametes (Pommerville, 1977). With the generation of pure male and female strains of *A. macrogynus* (Olson, 1984), parisin can be isolated. A series of general chemical tests have been completed on parisin (Pommerville and Olson, 1987a).

In chemotaxis bioassays, sperm cells or a supernatant solution from these cells stimulated attraction of female gametes. Sperm cells and supernatant were without effect using zoospores in the bioassay (Pommerville and Olson, 1987a). The stability of parisin was examined using chemical tests originally designed to study the stability of sirenin (see Machlis, 1958, for methods). The activity of parisin is maintained after freezing, boiling for 10 min, or autoclaving for 20 min (Pommerville and Olson, 1987a). However, the product of hot acid or alkaline conditions had no chemotactic activity.

Machlis (1958) demonstrated that sirenin could be extracted with ether. Following this protocol, parisin activity also remained associated with the ether fraction (Pommerville and Olson, 1987a). Thus, in these preliminary tests, the pheromone behaves similarly to sirenin.

PERSPECTIVES

The previous research activities point to the next stage in understanding the role of sirenin during chemotaxis in *Allomyces*. It is now possible to determine if pheromone receptors are present on the plasma membrane or in the cytoplasm of the sperm cells. In addition, we can identify the pathway(s) responsible for the influx of $[Ca^{2+}]$ into the sperm cytoplasm.

The studies discussed above demonstrate the ability to synthesize sirenin and a variety of active sirenin analogs. Therefore, we now can measure the

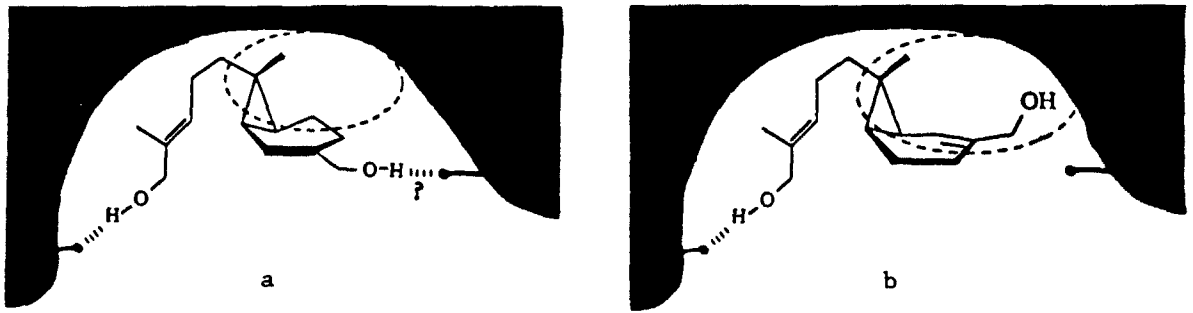

FIG. 4. A hypothetical model for the binding of *l*-sirenin (a) and the absence of binding for *d*-sirenin (b) with a presumptive sperm chemoreceptor. The *l*-enantiomer (a) would bind to the receptor by one or two hydrogen bonds (dashed lines) at the primary alcohol and the ring hydroxymethyl group, respectively. The bicyclic ring lies in a hydrophobic binding site of the receptor (dashed circle). *d*-Sirenin (b) would not bind due to inappropriate stereochemistry of the hydroxymethyl group.

binding of tritiated pheromone ([^{3}H]sirenin) or analogs to potentially small numbers of receptors. By using these radioligands, we can distinguish between actual receptors (specific binding sites) and nonspecific binding sites. In addition, since we have a better idea of the structure–activity relationships for sirenin, photoaffinity labeling can be carried out. Sirenin can be prepared with both a radiolabel and a photosensitive functional group to link covalently pheromone and receptor.

[^{3}H]Sirenin can be used to determine if the attractant enters the sperm cytoplasm. Uptake could involve the intact molecule or a labeled fragment. Absence of cytoplasmic radioactivity would suggest that stimulation of chemotaxis by sirenin requires Ca^{2+} as a second messenger system at the cell surface.

One process resulting from the binding of pheromones, hormones, or neurotransmitters to cell-surface receptors is the transduction of these signals into a change in cellular function or behavior. Ca^{2+} very often acts as a second messenger to transduce these signals (Rasmussen et al., 1985; Carafoli, 1987). How sirenin generates a transient rise in cytoplasmic Ca^{2+} needs to be determined. Because Ca^{2+} most often crosses the plasma membrane through channels, the existence of such channels on the sperm cell surface needs to be examined. Ligand binding to receptors can trigger potential-dependent channels that are responsible for the slow Ca^{2+} current or receptor-operated (mechanical) Ca^{2+} channels (Godfraind, 1985). Calcium and calmodulin antagonists can be used to identify the presence and types of calcium channels in *Allomyces*. Similar procedures have been used with other eukaryotic microbes, including the green alga *Chlamydomonas* and the protozoan *Paramecium* (Nultsch et al., 1986; Ehrlich et al., 1988), for the examination of calcium channels.

Finally, our findings point out some similarities between parisin and sirenin. Studies are in progress to determine the complete structure of parisin and to examine female gamete behavior in the presence of this attractant.

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BEHAVIORAL STUDIES INTO THE MECHANISM OF EUKARYOTIC CHEMOTAXIS

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Abstract—Recent behavioral studies designed to elucidate the mechanism of chemotaxis to cAMP in the eukaroyte *Dictyostelium discoideum* are reviewed. In these studies, ambae were analyzed by the newly developed, computer-assisted dynamic morphology system while (1) chemotaxing in a spatial gradient of cAMP, (2) responding to repeated temporal waves of cAMP in the absence of a spatial gradient in a Sykes-Moore chamber, and (3) responding to rapid shifts in cAMP concentration. It is demonstrated that eukaryotic amebae do indeed have the capacity to assess the direction of a temporal gradient, which indicates that they must have a "memory" system for this purpose. It is also demonstrated that amebae regulate behavior in spatial and temporal gradients of chemoattractant through changes in: (1) velocity; (2) frequency of pseudopod formation; and (3) frequency of turning. Analogies to the bacterial system are apparent.

Key Words—Chemotaxis, ameba, *Dictyostelium discoideum*, cAMP, temporal gradient, spatial gradient.

INTRODUCTION

Although the general mechanism of bacterial chemotaxis is reasonably well understood (Berg and Brown, 1972; Koshland, 1980; Boyd and Simon, 1982; Macnab and Koshland, 1972; Brown and Berg, 1974; Springer et al., 1977), the basis for eukaryotic chemotaxis remains elusive. Controversy still exists as to whether ameboid cells, such as those of *Dictyostelium* and polymorphonuclear leukocytes, employ a temporal or spatial mechanism to assess the direction of a spatial gradient of chemoattractant, even though significant progress has been achieved in defining the molecular components involved in sensory trans-

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duction. Without doubt, one of the major reasons for the slow progress in understanding how ameboid cells assess the direction of a chemical gradient is the prevailing attitude that behavioral studies are usually not definitive proof of mechanism and that all information on mechanism will ultimately emerge from molecular studies. Nothing could be further from the truth. In order to understand how chemotaxis really works in ameboid cells, three types of information must be used: (1) cell behavior; (2) biochemistry of receptors and sensory transduction; and (3) structural alterations in the cytoskeleton during the motile response. Behavioral studies can be designed that provide a very strong indication of mechanism (e.g., Zigmond et al., 1981; Varnum-Finney et al., 1978a,b) and that afford us with predictions concerning the mechanics of the sensory transduction machinery and the role of the cytoskeleton in the motile response (Wessels et al., 1988). In the discussion that follows, recent behavioral studies performed on individual amebae of the slime mold *Dictyostelium discoideum* will be reviewed. The studies demonstrate that amebae can assess the direction of a temporal gradient of chemoattractant in the absence of a spatial gradient and that amebae respond to temporal and spatial gradients of attractant in a fashion similar to bacteria, but substitute pseudopod formation for flagellar rotation in the motile response.

General Features of Aggregation in D. discoideum. When amebae of *D. discoideum* are washed free of nutrients and dispersed on an agar substratum, the cell carpet separates into distinct territories encompassing roughly 10^5 amebae. Within each territory, an alternating pattern of light and dark concentric bands can be visualized. Each light and dark bank originates in the center of the aggregation territory and travels outward to the edge of the territory without a significant change in width. Since band propagation exhibits the characteristics of a nondissipating wave, it is most easily described in wave parameters. The period varies from 10 min during early aggregation to 5 min during late aggregation (Alcantara and Monk, 1974; Durston, 1974). The velocity at which bands move remains constant throughout aggregation but is affected by cell density, varying from 43 μm/min for dense cultures (Gerisch, 1968) to 300 μm/min for cell monolayers (Alcantara and Monk, 1974; Gross et al., 1976). During aggregation, roughly 20 waves are propagated from the center to territory edge (Devreotes, 1982). At the very end of aggregation, cells in a territory separate into streams that channel rapidly into the final cell aggregate.

cAMP Wave. Bonner (1947) first demonstrated that aggregating amebae were attracted to a diffusible molecule emanating from aggregating cells. This "acrasin" was later identified as cAMP (Konijn et al., 1967, 1968). Utilizing a unique isotope dilution–fluorographic technique, Tomchik and Devreotes (1981) visualized waves of cAMP in aggregation territories. The waves were symmetrical and exhibited dimensions similar to those of cell movement bands (Devreotes et al., 1983). The concentration of cAMP in the peak and trough of

an average wave was calculated to be 10^{-6} M and less than 10^{-8} M, respectively.

Three components appear to be necessary for the genesis of cAMP waves (Shaffer, 1962): (1 autonomous secretion of pulses of cAMP from the aggregation center; (2 relay of the cAMP signal by outlying cells in the territory; and (3 degradation of the signal. Autonomous pulsing appears to begin in precocious cells in the population, and these cells have been considered by some investigators to be specialized (Ennis and Sussman, 1958), although evidence has been presented that suggests this is not always the case (Konijn and Raper, 1961). Once a pulse of cAMP is emitted from an aggregation center, a relay system ensures that the wave does not rapidly dissipate. In order to relay, outlying cells emit a signal of cAMP in response to the initial signal (Shaffer, 1975; Ross et al., 1975; Geller and Brenner, 1978; Devreotes et al., 1978), then remain insensitive to further stimulation during a refractory period to ensure that relay occurs only in an outward direction. To remove the signal so that amebae can respond to a new signal, cAMP is rapidly hydrolyzed by a membrane-bound phosphodiesterase (Dicou and Brachet, 1979; Kessin et al., 1979).

Directed Movement and the "Back of the Wave Paradox." An ameba must assess the direction of the aggregation center in order to move towards it in a directed fashion. In an aggregation territory, an ameba first encounters both a positive spatial (in relation to the aggregation territory center) and a positive temporal gradient of cAMP at the front of an outwardly traveling wave (Figure 1). In the positive spatial gradient, the concentration gradient of cAMP across the cell body is higher in the direction towards the aggregation center; in the positive temporal gradient, the concentration of cAMP in the immediate cellular environment increases with time. After the peak of a wave passes, an ameba encounters both a negative spatial and a negative temporal gradient in the back of the wave (Figure 1).

It has been assumed by many that an ameba simply assesses the spatial gradient in the wave in order to move in a directed fashion towards the aggregation center (e.g., Alcantara and Monk, 1974; Gerisch, 1968). Indeed, many of the original assays for chemotaxis employed relatively stable spatial gradients generated in agar substrates. These gradients were nonpulsatile, and groups of cells, when placed on the agar containing a spatial gradient of cAMP, exhibited a positive response in which single cells surged from the group in the direction of increasing concentration (e.g., Konijn et al., 1968; Mato et al., 1975; Varnum and Soll, 1984).

If directed movement is controlled exclusively by assessment of the spatial gradient, a paradox arises. Since it has been demonstrated that cells can change their direction of movement in a matter of seconds when challenged with a localized pulse of cAMP (Futrelle et al., 1982; Swanson and Taylor, 1982), amebae should change direction on average 180° when they experience the

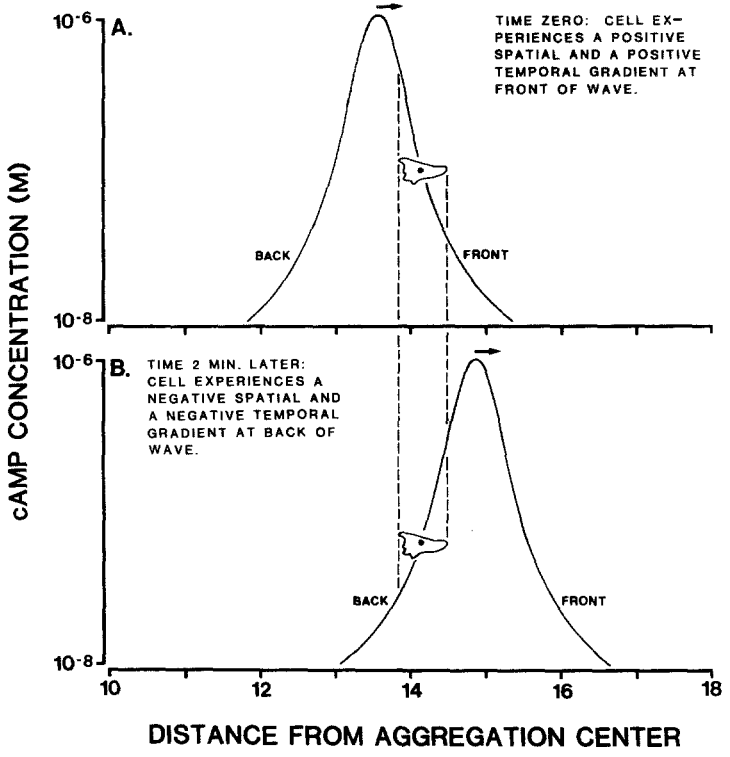


FIG. 1. Diagram of a wave of cAMP passing across a cell in a natural aggregation territory. The peak of the wave has been estimated to be 10^{-6} M cAMP and the trough less than 10^{-8} M. The front of the wave covers 3.5 min and the back 3.5 min. Distance from the aggregation center (source of wave) is in arbitrary units. The arrow at top of the wave indicates the direction of the moving wave. See text for a discussion of the wave paradox.

reversed spatial gradient at the back of the wave. A change in direction would negate net progress towards the aggregation center. They apparently do not. Therefore, a mechanism must exist for the inhibition of turning.

There are at least four possible mechanisms that may play a role in inhibiting turning. First, it is possible that the high concentration of cAMP that cells encounter at the peak of a wave may suppress turning and that a recovery period would be similar in length to the back of the wave. Second, the increasing temporal gradient in the front of the wave may inhibit turning by an adaptation process in which cells adapt through the increasing temporal gradient in the front of the wave but become unresponsive to the decreasing temporal gradient at the back of the wave. Third, the decreasing temporal gradient in the back of

the wave may directly inhibit turning. This mechanism requires that a cell be capable of measuring the direction of a temporal gradient. Unlike the prior alternative, adaptation in the increasing phase is not a component in this mechanism. Finally, the establishment of polarity in a cell during the front of the wave may retard a change in direction during the back of the wave. In the first three alternatives, inhibition could be accomplished by: (1) inhibiting the rate of motility; (2) inhibiting the capacity to read a spatial gradient; (3) decreasing the frequency of turning; or (4) decreasing the degree of turning.

Although it has been convincingly demonstrated that individual *Dictyostelium* amebae can assess the direction of a spatial gradient (Varnum and Soll, 1984), it is not known how they do so. The most obvious mechanism for assessing a spatial gradient would be for the ameba to measure the concentration differential across the entire cell body. This represents a spatial mechanism. However, if one employs the diffusion equation to calculate the concentration differential across a cell body in a Zigmond chamber, the percent difference between the ends of the cell is no more than 1–2%. If a spatial gradient is measured along the length of a pseudopod, the concentration differential would be far smaller.

Alternatively, amebae may assess a spatial gradient by a temporal mechanism comparable to that of a bacterium. By moving through a positive spatial gradient, a cell would experience an increase in attractant with time; by moving through a negative spatial gradient, a cell would experience a decrease in attractant with time. The capacity to assess the direction of a temporal gradient requires that the cell have a form of memory. It must know at any moment whether the concentration of attractant at a previous moment was higher or lower, a comparison which is probably receptor mediated (Green and Newell, 1975; Klein and Juliani, 1977; Zigmond, 1977).

Methods for Analyzing Cell Behavior in Spatial and Temporal Gradients of cAMP. In order to analyze the behavior of individual amebae in relatively stable spatial gradients and in increasing and decreasing temporal gradients of chemoattractant, we employed two chamber systems. The first, originally developed by Zigmond (1977), consists of a Plexiglass bridge bounded by two troughs. Amebae on the bridge or attached to the overlying cover slip experience a spatial gradient that flattens with time. Relatively accurate calculations of concentration at a cell location for a particular time can be made employing the diffusion equation (Varnum and Soll, 1984). The second chamber consists of two glass walls separated by a rubber O ring. In this Sykes-Moore chamber, cells attached to the glass wall are perfused with solution at rates high enough to ensure that cells cannot condition their microenvironment (Herman and Soll, 1984; Staebell and Soll, 1985). This chamber is excellent for generating temporal gradients in the absence of a spatial gradient and for mimicking the temporal dynamics of a natural wave (Varnum et al., 1985).

To monitor the behavior of cells in these chambers, the microscope field is continuously videorecorded. Originally, we analyzed the recordings with a digitizing pad attached to an IBM-PC programmed to calculate several simple parameters of motion. This was an extremely slow process. This process was accelerated at least 20-fold with a SUN II-driven Expertvision System purchased from Motion Analysis Corp. (Santa Rosa, California). This system allowed us to monitor up to 40 cells in parallel for linear velocity, angular velocity, turning, and directionality, with rapid statistical packages for each parameter. However, this system did not provide information on the morphological changes accompanying a cell's behavior. We therefore developed the dynamic morphology system (DMS), which can measure up to 30 parameters of cell movement and shape as frequently as every thirtieth of a second for up to 40 cells (Soll, 1988; Soll et al., 1988a,b). Parameters monitored by DMS include velocity, directional change, turns per unit time, angular velocity, width, length, roundness, polarity of pseudoped formation, frequency of pseudopod formation, expansion area per unit time, contraction area per unit time, concavity, and convexity (Soll et al., 1988a,b).

Constant Concentrations of cAMP Depress Cell Motility. The average rate of motility (measured as linear velocity and averaged over a 21-min period) is similar in buffer solution lacking cAMP and in buffer solution containing cAMP at 10^{-10} and 10^{-8} M, the estimated concentrations at the trough of the natural wave (Varnum and Soll, 1984). However, above 10^{-8} M cAMP, velocity is depressed in a concentration-dependent fashion, reaching a plateau of roughly 35% maximum velocity (measured in buffer alone) at 10^{-5} M cAMP (Figure 2). This response is observed in both a Zigmond chamber and a Sykes-Moore chamber at high perfusion rate. Therefore, no constant concentration of cAMP stimulates motility, and constant concentrations above 10^{-8} M actually depress motility (Varnum and Soll, 1984).

A rapid increase in cAMP concentration depresses motility, increases directional change, and causes disoriented pseudoped formation; this response exhibits adaptation. Shifting the concentration of cAMP from 0 M to concentrations ranging from 10^{-8} to 10^{-6} M in a Sykes-Moore perfusion chamber (shift time 15.8 sec) results in an immediate reduction in velocity (Varnum-Finney et al., 1988; see data for a representative cell in Figure 3A). After shifting from 0 M to either 10^{-8} or 10^{-7} M cAMP, velocity is inhibited, then rebounds, exhibiting a standard adaptation response (Figure 3A). However, a shift from 0 M to 10^{-6} M cAMP (the estimated peak concentration of a natural wave) results in inhibition without adaptation. A shift from 0 M to concentrations ranging from 10^{-8} to 10^{-6} M cAMP also results in: (1) an increase in directional change (Figure 3B); (2) an increase in roundness (Figure 3C); (3) a decrease in length (Figure 3D); and (4) a depression in positive flow (Figure

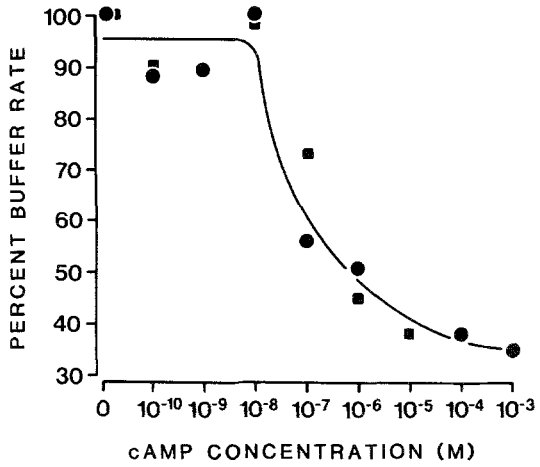


FIG. 2. The average rate of cell motility (presented in percent of the buffer rate) as a function of cAMP concentration. Each point represents the average rate measured over a period of 20 min for 50 cells. The filled circles represent amebae in homogenous solutions of cAMP (nongradient conditions) at the respective concentrations. The filled squares represent amebae in gradients of cAMP. In the latter case, the average concentration of cAMP at the position of the cell body was calculated by the diffusion equation. Details of these measurements are presented in Varnum and Soll (1984).

3F). In the case of a shift to 10^{-8} M cAMP, all parameters return to near-normal after adaptation, but in the case of a shift to 10^{-6} M cAMP, cells continue to exhibit these characteristics in the absence of adaptation. The increase in directional change following a shift reflects a decrease in cellular polarity and an increase in the number of expansion zones (filled areas) and contraction zones (hatched areas) visualized in "difference pictures" (Figure 4). Prior to a shift to 10^{-8} M cAMP (panels 13.17m to 14.67m in Figure 4), cells exhibit an elongate morphology and directional persistence (note direction of vectors). After a shift (panels 15.00m to 18.17m in Figure 4), the cells exhibit disorganized behavior, including the loss of polarity and multiple expansion zones. After adaptation, the cells regain their elongate shape, oriented behavior, and singular optical expansion zone.

It should be noted that most studies into the receptor-mediated effects of cAMP on cellular physiology, relay, and the cytoskeleton follow the general experimental format of a cAMP pulse or shift. However, rapid shifts lead to the inhibition of motility and most probably are never experienced by an ameba in a natural aggregation territory. Indeed, the increase in the cAMP concentration at the front of a natural wave occurs 14 times more slowly than it does

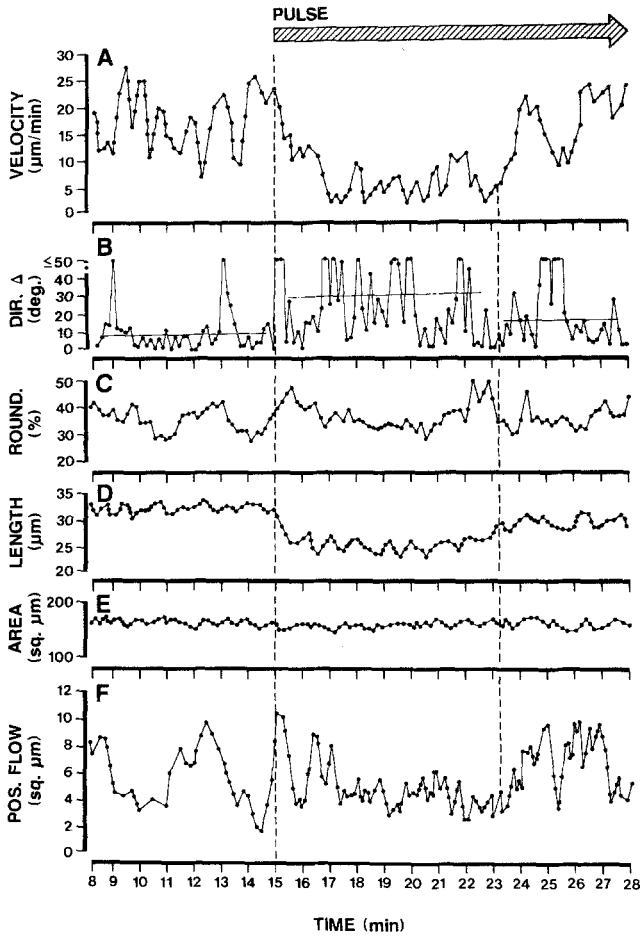
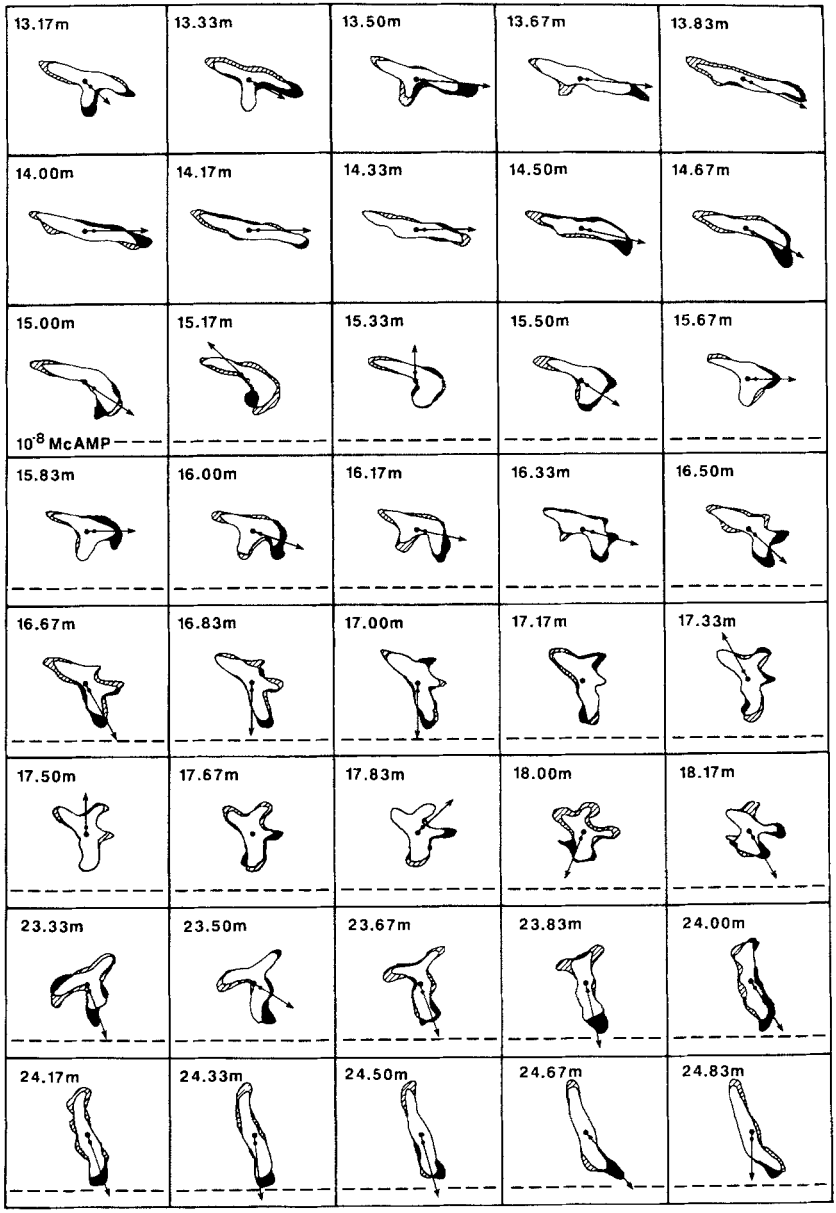


FIG. 3. Parameters monitored by the dynamic morphology system of a cell in a Sykes-Moore chamber perfused for 15 min with buffer lacking cAMP, then for 15 min with buffer containing 10^{-8} M cAMP. (A) velocity, measured as instantaneous velocity; (B) directional change; (C) roundness parameter (a perfect circle provides a roundness parameter of 100% and a straight line a parameter of 0%); (D) length; (E) area; and (F) positive flow (this measurement was assessed by differencing every 5 sec; see legend in Figure 4). Points are plotted for the final 7 min in buffer and initial 13 min after the shift. The first vertical dashed line represents the onset of the shift, and the second vertical dashed line the estimated time of adaptation. The horizontal hatched line at the top of the figure represents the time after shift.

during a shift in a Sykes-Moore chamber and probably two orders of magnitude more slowly than a pulse of cAMP administered to a rotating suspension culture.

Effects on Cell Behavior of Temporal Waves of cAMP that Mimic Temporal Dynamics of Natural Waves. Using a Sykes-Moore perfusion chamber, the temporal dynamics of natural waves can be mimicked in the absence of a spatial gradient (Varnum et al., 1985). The generated gradients are diagramed in Fig. 5E. In Figure 5A–D, the tracks of four individual cells perfused with repeated "temporal waves" of cAMP are presented; in Figure 5E, the interval velocities are presented for a single cell treated with repeated temporal waves. In almost every case, the velocity during the increasing and decreasing phase of the first wave was depressed. However, in subsequent waves, cell motility was stimulated in the increasing phase and dramatically reduced in the decreasing phase (Figure 5E). When a concentration range was selected for which the highest concentration was not inhibitory when constant, the rate of motility was stimulated during the increasing phases of waves 2–4 to levels slightly higher than the basal rate. These results demonstrate: (1) an ameboid cell can assess the direction of a temporal gradient in the absence of a spatial gradient and modify its velocity accordingly; (2) although no constant concentration of cAMP can stimulate motility, an increasing temporal gradient will do so; and (3) a memory system exists that allows a cell to compare the present concentration of an attractant with a prior concentration.

The results presented in Figure 5 suggest that the direction of the temporal gradient at the front and back of a wave regulates the rate of motility and therefore plays a role in the net movement of cells towards the aggregation center. The direction of a temporal gradient may affect pseudopod formation, cell shape, turning, and cell polarity, and in so doing enhances the effect on velocity. The results of studies on pseudopod formation during waves 2, 3, and 4 demonstrate that the direction of a temporal gradient does indeed affect pseudopod extension and turning (Table 1). During the first 35 sec of the increasing portions of waves 2–4, the mean frequency of new pseudopod formation was 15/10 min (± SD 7.6) and the mean frequency of turns resulting from new pseudopod formation was 10/10 min (± SD 7.5); therefore, 67% of new pseudopods resulted in turns. In contrast, during the remaining 17.5 sec of the increasing portion of the wave, the mean frequency of new pseudopod formation was 5.2/10 min (± SD 2.4) and only 15% resulted in turns. During the first 35 sec of the decreasing portion of the wave, mean frequency was 7.5 (± SD 7.0) and only 33% resulted in turns. The frequency and percent turns was roughly unchanged during the last 175 sec of the decreasing portion of the wave. Therefore, during the first


13.17m
13.33m
13.50m
13.67m
13.83m
14.00m
14.17m
14.33m
14.50m
14.67m
15.00m
15.17m
15.33m
15.50m
15.67m
10-8 McAMP
15.83m
16.00m
16.17m
16.33m
16.50m
16.67m
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17.00m
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20% of the increasing phase of a temporal wave, the frequency of pseudopod formation and the proportion of pseudopodia that results in turns are high; however, both frequency and turning rapidly decrease and remain low during the last 80% of the increasing phase. During the entire decreasing phase, both the frequency of pseudopod formation and the proportion giving rise to turns remain at an intermediate level, very close to the values observed at a constant concentration of 10^{-8} M cAMP (Table 1).

Behavior of Cells in Spatial Gradients of cAMP. Individual amebae exhibit a strong positive chemotactic response to relatively stable gradients of cAMP generated in a Zigmond chamber. The chemotactic index is maximal when the average concentration at the position of the cell body is 10^{-9}–10^{-8} M cAMP. Gradients that result in average concentrations at the cell body above 10^{-8} M result in reduced chemotactic indices in the chamber. It should be noted that amebae respond maximally to concentrations that approximate the K_D of the high-affinity cAMP-binding site of *D. discoideum* (Green and Newell, 1975). Similar results were obtained in the response of leukocytes to pentapeptide chemoattractants (Zigmond and Sullivan, 1979).

When amebae are migrating towards the source in a gradient of cAMP (high chemotactant index), they move faster, extend pseudopodia at a dramatically reduced frequency, and turn far less often than when migrating away from the source (Figure 6A–C, respectively). For cells oriented at an angle to the source, the same number of pseudopods form in the correct direction (towards the source) as in the incorrect direction (away from the source), but cells more frequently turn in the direction of pseudopods oriented in the correct direction than in the direction of pseudopods oriented in the incorrect direction (Varnum-Finney et al., 1988b). These results suggest that a decision-making mechanism may be localized in the pseudopodial region (anterior half) of polarized cells.

Making Sense of Behavioral Studies. The behavioral studies reviewed in

FIG. 4. Difference pictures generated by the dynamic morphology system of a cell (the one analyzed in Figure 3) in a Sykes-Moores chamber perfused for 15 min in buffer, then 15 min in 10^{-8} M cAMP. In the genesis of each difference picture, the cell outline in the frame noted in the upper left-hand corner of each panel (time in minutes) is superimposed on the cell outline on a frame 10 sec earlier. The expansion zones are filled and the contraction zones hatched. To indicate direction of translocation between frames, an arrow is drawn from the centroid of the earlier frame (large filled circle) through the centroid of the later frame (small filled circle). The shift to 10^{-8} M cAMP begins at 15 min (panel 15.00m) and is noted in subsequent panels by a dashed horizontal line. This particular cell adapted between 23 and 24 min with an increase in velocity (see Figure 3A). This adaptation coincides with an elongation of cell shape (panels 23.67m to 24.17m).

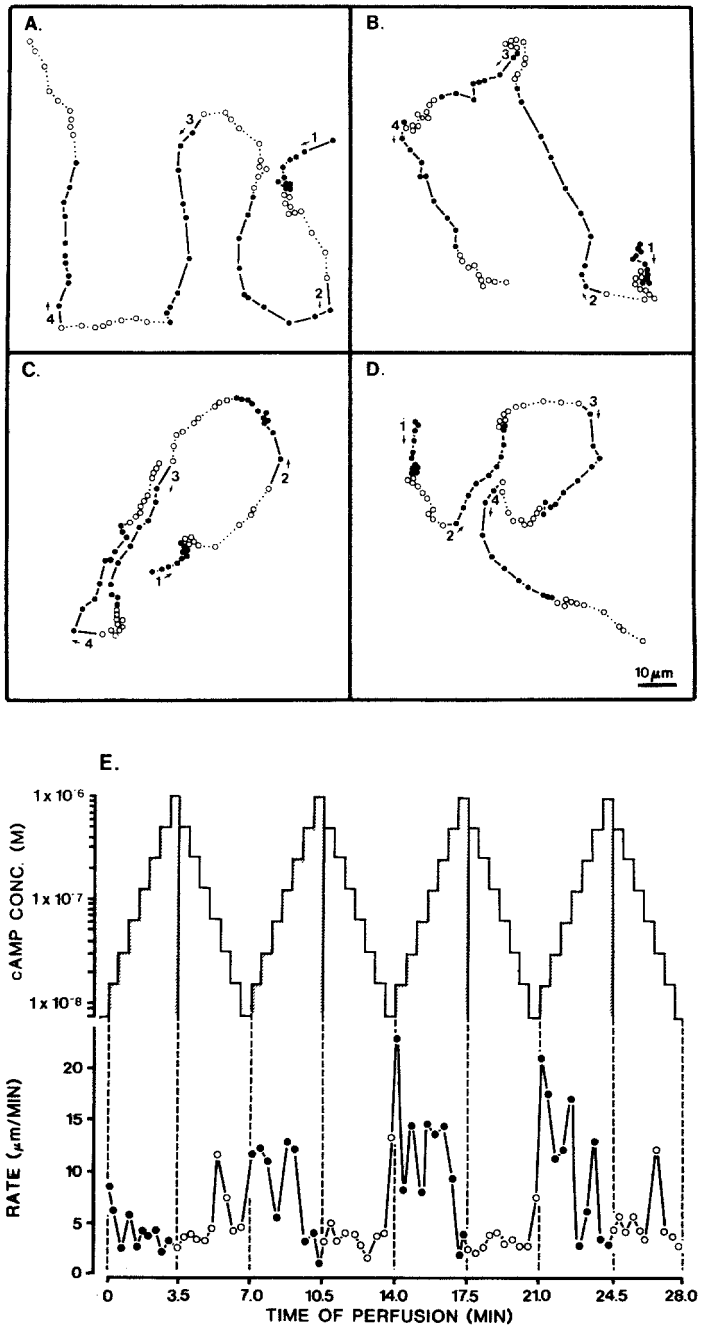
A.
B.
C.
D.
10 µm
E.
cAMP CONC. (M)
1 x 10⁻⁶
1 x 10⁻⁷
1 x 10⁻⁸
RATE (µm/MIN)
0
5
10
15
20
0
3.5
7.0
10.5
14.0
17.5
21.0
24.5
28.0
TIME OF PERFUSION (MIN)

TABLE 1. COMPARISON OF MEAN FREQUENCY OF PSEUDOPOD FORMATION AND OF TURNING AND PERCENTAGE OF NEW PSEUDOPODS PRODUCING A TURN FOR AMEBAE TREATED WITH INCREASING AND DECREASING PHASES OF A SIMULATED TEMPORAL WAVE OF cAMP[a]

Gradient condition	No./10 min		Proportion of pseudopods that produce turns (%)
	Mean frequency of new pseudopod formation ± SD	Mean frequency of turning ± SD	
Increasing phase			
First 35 sec	15.0 ± 7.6	10.0 ± 7.5	67
Last 175 sec	5.2 ± 2.4	0.8 ± 1.7	15
P value	$p < 0.001$	$p < 0.01$	
Decreasing phase			
First 35 sec	7.5 ± 7.0	2.5 ± 4.6	33
Last 175 sec	7.7 ± 2.5	3.3 ± 1.8	43
P value	NS	NS	
Constant cAMP, 10^{-8} M; 12 min	5.8 ± 3.9	3.2 ± 2.1	55

[a] Details of simulated waves are presented in Figure 5. The means were calculated from the combined data from eight independent cells. Data were averaged for the last three of four simulated waves. The significance of the difference between the value for the first 35 sec and the value for the last 175 sec of each parameter was assessed by the *P* value test; NS, not significant. Details are presented in Varnum-Finney et al. (1987b).

this discussion can be separated into two categories: (1) those in which behavior is assessed before and after a single rapid shift in attractant concentration, and (2) those in which different aspects of the natural wave are mimicked. Both are important since they provide information on different aspects of chemotaxis. Rapid shifts have been employed in most analyses of receptors and receptor-mediated changes, including sensory transduction pathways and cytoskeletal changes. It is, therefore, important that we have as detailed a description of the behavioral effects as we do of the physiological effects, since the behavior must

FIG. 5. The behavior of amebae perfused with four temporal waves of cAMP that mimic the temporal dynamics of natural waves in the absence of spatial gradients. (A–D) Tracks of individual amebae during four temporal waves. Centroids of each cell were determined at 21-sec intervals during the increasing (●) and decreasing (○) phase of each wave. Concentric waves are numbered 1–4. The arrows represent the direction of movement. (E) The temporal dynamics of simulated waves 1–4 and the velocity of centroid translocation (calculated for each 21-sec segment of a single cell track) plotted during the increasing (●) and decreasing (○) phases of the four waves. Details of these data are presented in Varnum et al. (1985).

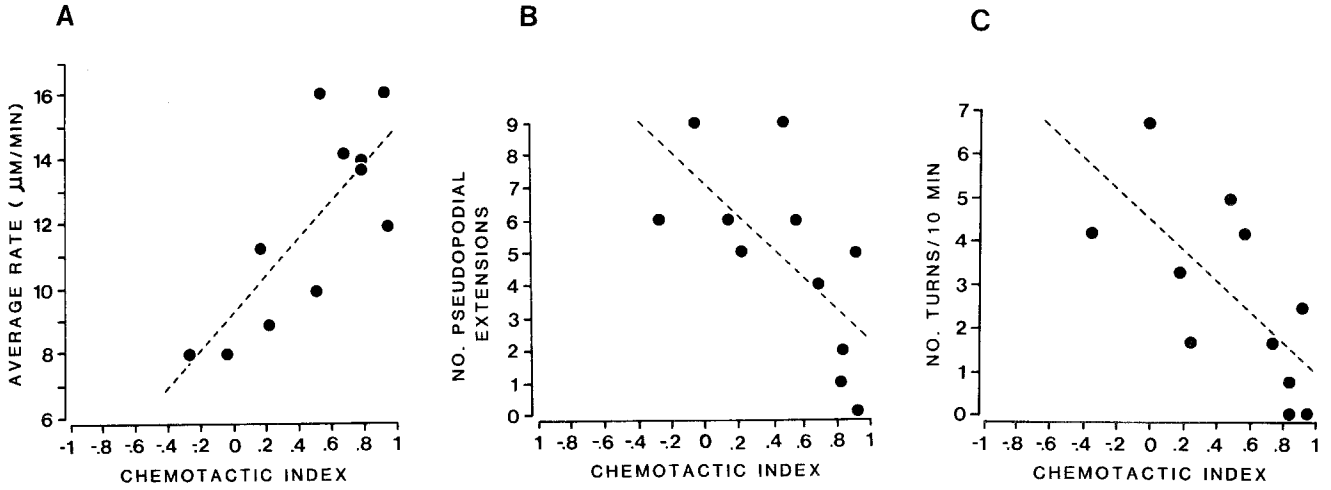

FIG. 6. Velocity (A), frequency of pseudopod formation (B), and frequency of turning (C) as functions of the chemotactic index. (A) The velocity was averaged for each cell over a 12-min period; (B) the number of pseudopodial extensions was measured for each cell for a 12-min period; (C) the number of turns was measured for each cell for a 12-min period. Cells were monitored during a 12-min period in a Zigmond chamber containing 10^{-7} M cAMP in one trough (source) and buffer in the other (sink). Chemotactic index was calculated by dividing directional distance (net distance toward source) by total distance (Varnum and Soll, 1984). Dashed lines represent regression lines calculated for the data of points.

provide a context for interpreting the physiology. Unfortunately, few researchers have attempted to assess physiological parameters in cells that are actively chemotaxing in a spatial gradient of attractant or in cells treated with temporal waves mimicking the temporal dynamics of natural waves. The differences we have demonstrated in the motile response of cells treated with a shift, cells treated with repeating temporal waves, and cells actively assessing a relatively stable spatial gradient argue very strongly for a reassessment of physiological parameters under the latter two sets of conditions.

Perhaps the most important experiments that we have reviewed are those in which the cellular response to repeating temporal waves of cAMP is examined in a Sykes-Moore chamber. These experiments demonstrate that *Dictyostelium* amebae are capable of assessing the direction of a temporal gradient of cAMP and adjusting: (1) the rate of centroid translocation (Varnum et al., 1985); (2) the frequency of pseudopod formation; and (3) the frequency of turning into, or retracting, lateral pseudopods (Varnum-Finney et al., 1987a). It is not clear why cells are not stimulated by the first of four temporal waves. However, it is clear that in the second wave motility is stimulated submaximally during the increasing phase and depressed during the decreasing phase and that in the third and fourth waves motility is stimulated maximally during the increasing phase and in the decreasing phase is depressed to the same extent as in the first wave (Varnum-Finney et al., 1988). The transition from the decreasing to increasing phase of consecutive waves results in a temporary stimulation of pseudopod formation and turning. As the increasing phase continues, both pseudopod formation and turning frequency are suppressed, which facilitates translocation with directional persistance. Extrapolating from the behavior observed in temporal gradients in the Sykes-Moore chamber to behavior expected in a natural wave, one would expect rapid and directionally persistent translocation at the front of the wave and depressed translocation and a higher frequency of turning at the back of the wave. Recent analyses of single cell behavior in natural aggregation territories indicate that this prediction is correct (Wessels and Soll, in preparation).

Temporal wave experiments may explain velocity, pseudopod frequency, turning frequency, and persistence, but they do not necessarily explain how cells orient towards the source of attractant. Experiments in spatial gradients indicate how this is achieved. When a cell is oriented in the wrong direction in a spatial gradient (negative chemotactic index), it moves slowly and exhibits higher frequencies of pseudopod formation and turning than cells oriented in the correct direction (Varnum-Finney et al., 1987b). This mechanism will lead to orientation, since it will trap cells as they orient in the correct direction. These results determine behavior as a function of chemotactic index, but they do not discriminate between temporal and spatial mechanisms. Two sets of observations, however, suggest that a temporal mechanism is functioning in a

spatial gradient. First, a cell oriented at an angle to the source produces pseudopods towards the source as frequently as it does away from the source, but turns two to three times more frequently in the correct direction than in the incorrect direction, suggesting that discrimination of direction is localized in the extending pseudopod at the anterior end of the cell. Second, if one mimics the increasing temporal gradient of cAMP that would be generated by a cell moving towards the source (chemotactic index of +1) in a Zigmond chamber, the frequency of pseudopod formation and the frequency of turning is depressed (Varnum-Finney et al., 1987a). Therefore, an ameba can assess the direction of a temporal gradient generated when it moves in a relatively stable spatial gradient.

We have begun to elucidate a chemoresponse system in eukaryotic amebae that includes a temporal mechanism analogous to that of bacteria for assessing the direction of a temporal change in attractant. Instead of affecting flagellar rotation (Macnab and Koshland, 1972; Berg and Brown, 1972), the motile response of an ameba involves cell polarity, pseudopod formation, turning, and pseudopod retraction. Although the behaviors in amebae are effected through the reorganization of the intracellular cytoskeleton, cytoplasmic flow, and membrane formation, the end results are similar to the bacterial system and no doubt represent the parallel evolution of temporal mechanisms in bacteria and eukaryotes. However, it should be noted that proving amebae have the capacity to assess the direction of a temporal gradient does not prove that this mechanism is uniquely employed in the chemotactic process (Varnum-Finney et al., 1987b). Considering the complexity of the chemotactic responses described in the studies reviewed here, it would not be surprising if both temporal and spatial mechanisms are involved in the chemotactic response of ameboid cells. Further behavioral studies of wild-type and mutant cells (Soll et al., 1988b; Wessels et al., 1988) will be instrumental in describing the mechanisms involved.

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SEQUESTRATION OF DISTASTEFUL COMPOUNDS BY SOME PHARMACOPHAGOUS INSECTS

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Abstract—Several pharmacophagous insects have been shown to sequester specific kairomonal substances or their derivatives in their body tissues. Turnip sawflies, *Athalia rosae*, visit a plant, *Clerodendron trichotomum* (Verbenaceae), and feed voraciously on the leaf surface. Clerodendrins were characterized as the potent phagostimulants for *A. rosae* adults. The insect sequesters some of the analogs and becomes extremely bitter on its body surface. Some chrysomelid leaf beetles associated with cucurbitacins were found to store high concentrations of these bitter principles in their body. South American polyphagous beetles, *Diabrotica speciosa* and *Cerotoma arcuata*, are strongly arrested by root components from the cucurbit plant, *Ceratosanthes hilariana*, and selectively accumulate 23,24-dihydrocucurbitacin D, effectively gaining bitterness. Similarly, four species of Asian pumpkin leaf beetles belonging to the genus *Aulacophora* were shown to sequester the same compound in body tissue as the major bitter principle. Three phenylpropanoids closely related to methyl eugenol were found to accumulate in the rectal glands of the male Oriental fruit fly, *Dacus dorsalis*. One of the rectal gland components, 2-allyl-4,5-dimethoxyphenol was shown to be released in the air during courtship. In all of these cases, selectively sequestered compounds strongly deterred feeding by some predators, thus serving as allomones in this context. Kairomonal and pheromonal functions linked with allomonal sequestration by pharmacophagous feeding has also been suggested.

Key Words—Sequestration, pharmacophagy, kairomone, allomone, *Athalia* spp., *Diabrotica* spp., *Dacus dorsalis*, clerodendrin, cucurbitacin, methyl eugenol.

INTRODUCTION

A number of insects are strongly associated with specific chemicals produced by plants that are not their own host. Strong associations between pyrrolizidine alkaloids and several lepidopterous insects such as danaid butterflies (Meinwald et al., 1969; Boppré et al., 1978; Boppré, 1978) and arctiid moths (Conner et al., 1981; Schneider et al., 1982) have been extensively studied. By sequestering these plant metabolites, the insects gain protection from predators and precursors for the male pheromones (Boppré, 1986). These insects gather the chemicals not from their primary host, but from totally unrelated plants. Boppré (1984) proposed the redefined term "pharmacophagy" for this particular type of insect–plant association. However, very few additional examples of such interactions between insects and foreign plant chemicals have been discovered (Boppré, 1984, 1986; Ferguson and Metcalf, 1985).

We have studied three cases of pharmacophagous associations among important agricultural pest insects, including the turnip sawfly (Hymenoptera) –clerodendrins, dibroticite leaf beetles (Coleoptera) –cucurbitacins, and the Oriental fruit fly (Diptera) –phenylpropanoids (Figure 1). In all of these cases,

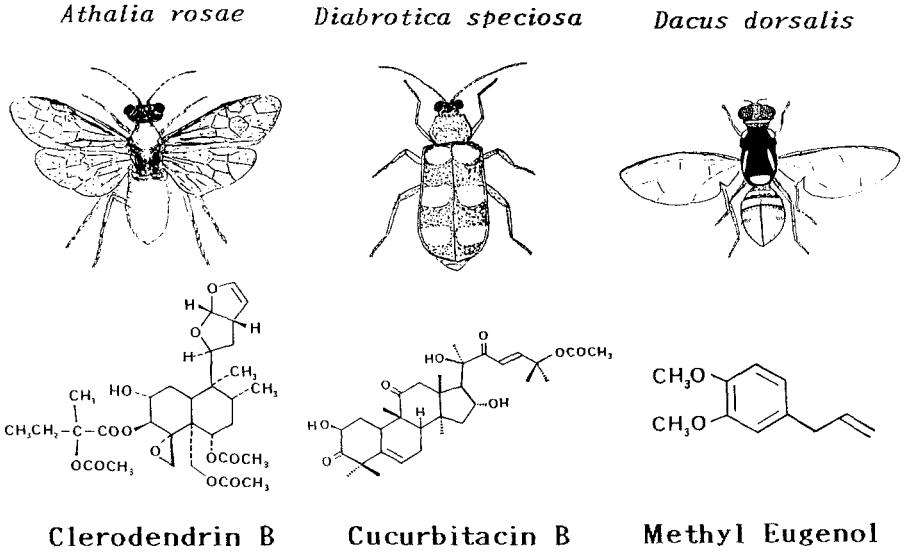

FIG. 1. Pharmacophagous insects and kairomonal substances associated with the insects. Left: A turnip sawfly, *Athalia rosae*, is stimulated to feed on leaves of *Clerodendron trichotomum* in response to the phagostimulant, clerodendrin B. Middle: *Diabrotica speciosa* and other related leaf beetles are stimulated by cucurbitacin B contained in various cucurbit plants. Right: Males of the Oriental fruit fly, *Dacus dorsalis* are attracted to methyl eugenol, and ingest the essential oil.

the insects are strongly stimulated to feed on the kairomonal substances and sequester the specific chemicals (or their derivatives), thereby utilizing them as allomones. Some pheromonal effects linked with pharmacophagous feeding are also suggested in some cases. Here, we describe the identification of the chemicals sequestered by pharmacophagous insects and the ecological significance of these substances.

TURNIP SAWFLY–CLERODENDRIN ASSOCIATION

A turnip saw fly, *Athalia rosae* (*ruficornis*), feeds exclusively on the plant family Cruciferae (Abe, 1988). The larvae infest many kinds of cruciferous vegetables, but the adult insects do not feed on the larval hosts. However, the adult flies frequently visit a shrubby plant, *Clerodendron trichotomum* (Verbenaceae), which is entirely unrelated to their host-plant family Cruciferae (Kitano, 1988). The sawflies eagerly feed on the leaf surface. Both sexes are attracted to the plants, and mating behavior commonly can be seen on the leaves. When the leaf surface was mechanically disturbed, an extremely bitter substance was exuded. The bitter substance appeared to serve as a phagostimulant for *A. rosae*.

Identification of Kairomones from C. trichotomum. The potent feeding stimulants were effectively extracted by quickly rinsing the leaves of *C. trichotomum* with a mixture of acetone and hexane (2 : 1) (Nishida et al., 1989). When the extract was dispensed on a piece of filter paper (8 × 8 mm^2), the sawflies eagerly fed on the surface if contacted with their mouthparts (Figure 2). Using this simple behavioral bioassay, two kairomonal substances, clerodendrin B and D, were characterized (Nishida et al., 1989). Clerodendrin B was previously identified from this plant together with a major component, clerodendrin A, as insect antifeedants (Kato et al., 1971, 1972). Although the plane structure of D is identical to that of ajugapitin (Hernández et al., 1982), the configuration of both D and ajugapitin at the 2-methylbutyryl moiety has yet to be determined (Figure 3). Clerodendrins B and D were strongly stimulating to both sexes of *A. rosae*, but clerodendrin A did not induce any feeding responses from the insects in spite of its predominance in the leaf extracts (Nishida et al., 1989).

Sequestration of Clerodendrins. It was noted that a large portion of the wild *A. rosae* population possesses an extremely bitter taste, while laboratory-reared adults did not taste bitter. The indoor females that were fed with *Clerodendron* leaves quickly gain the bitterness on their body surface. *Clerodendron*-fed sawflies were significantly rejected by a lizard, *Japalura polygonata* (Johki and Nishida, unpublished data). Figure 4 shows the thin-layer chromatograms (TLC) of the extracts from the bodies of *A. rosae* females fed (upper) and not fed (lower) *Clerodendron* leaves for at least three days before extraction and then fed with 10% sucrose solution. The extracts of *Clerodendron*-fed

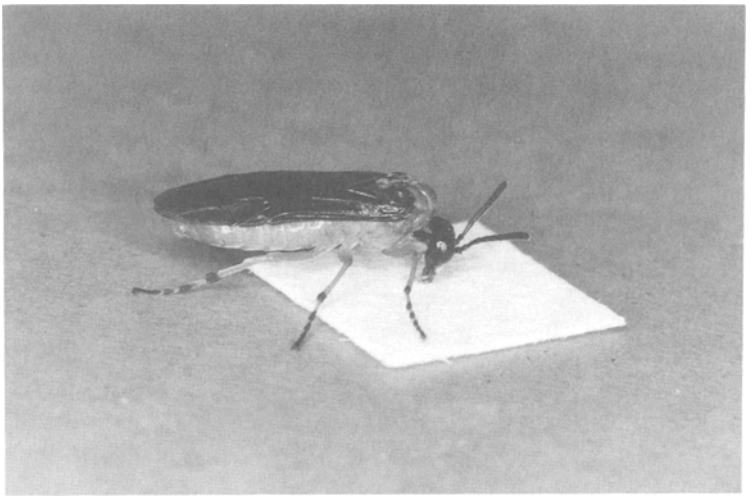

FIG. 2. Feeding test of turnip sawflies to an extract of *Clerodendron trichotomum*. A female insect is feeding on a square piece of filter paper treated with the sample.

females exhibited three clear spots (*Rf* = 0.61, 0.50, and 0.05) which were absent in those of unfed females. All three of these components, purified by HPLC (Nucleosil 100-5, 300 × 8 mm ID, 60% methyl acetate in hexane, 2 ml/min), showed a bitter taste. One of the components (TLC: *Rf* = 0.61) was identified as clerodendrin D from the retention time (*Rt* = 10.6 min) and from its diagnostic mass spectrum (MS: M^+, *m/z* 550) and proton NMR spectrum.

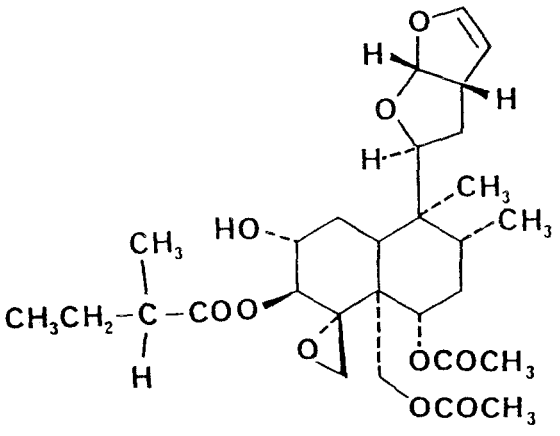

FIG. 3. Structure of clerodendrin D, a bitter substance sequestered by *Athalia rosae*.

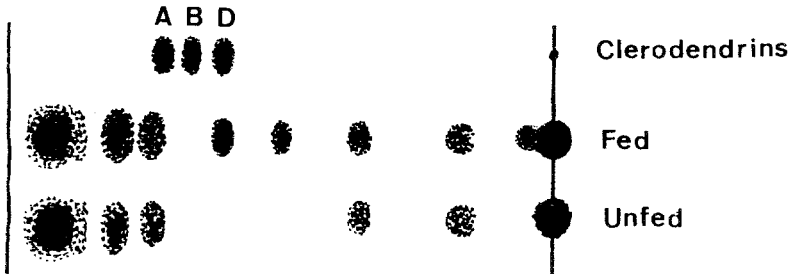

FIG. 4. Thin-layer chromatograms of clerodendrin A, B, and D (upper), body extracts of *Athalia rosae* females that were fed (middle) and not fed (lower) with *Clerodendron* leaves (HPTLC silica gel $60F_{254}$, nano-TLC, Merck, developed with benzene–ethyl acetate, 1 : 1, I_2 coloration).

Two other bitter components also appeared to be clerodendrin analogs (structural elucidations are in progress). The yield of clerodendrin D was estimated to be 8 μg/female. No significant amounts of clerodendrin A or B were present in the extract, even though clerodendrin D is a very minor component in the leaf extract. The external bitterness of *Clerodendron*-fed *A. rosae* bodies implies that a portion of the substances is being deposited on the cuticular surface. Sequestration of these components by the male insects has not yet been confirmed. Clerodendrin D deters birds, and its possible role as allomone will be discussed in the later section. In addition, females that have sequestered clerodendrins become more attractive to the males (Nishida, unpublished data), suggesting some pheromonal function involved in their courtship sequence.

CHRYSOMELID LEAF BEETLES-CUCURBITACIN ASSOCIATION

Many polyphagous chrysomelid leaf beetles (mainly diabroticites) show strong affinity to cucurbit plants that are not their ordinary hosts for the larvae. Curcurbitacins have been shown as the characteristic kairomones for these beetles (Chambliss and Jones, 1966a; Metcalf, 1979, 1986; Nishida et al., 1986). Above all, cucurbitacin B and D are extremely potent stimulants for several diabroticites (Chambliss and Jones, 1966b; Metcalf et al., 1982). These two compounds are present most commonly in various kinds of cucurbit plants and are known to be the source of the bitterness (Lavie and Glotter, 1971; Metcalf, 1979). It has been demonstrated that *Diabrotica* spp. accumulate the bitter principles by feeding on cucurbit plants during adulthood, thus effectively deterring feeding of a mantid (Ferguson and Metcalf, 1985). The fate of cucurbitacins in the bodies of the adult beetles has been examined by artificially feeding them with ^{14}C-labeled curcurbitacin B (Ferguson et al., 1985). The cucurbit plants,

however, contain a complex mixture of cucurbitacin analogs in varying quantity (Lavie and Glotter, 1971). The wild chrysomelid leaf beetles appeared to accumulate only a limited number of analogs, which are not always the major analogs in the plants (Nishida et al., 1990).

Sequestration of Cucurbitacins by Two Brazilian Leaf Beetles. Nishida et al. (1986) identified cucurbitacin B and 23,24-dihydrocucurbitacin B from the root of *Ceratosanthes hilariana* (Cucurbitaceae) as the major feeding stimulants of two Brazilian leaf beetles, *Diabrotica speciosa* and *Cerotoma arcuata*. By feeding the roots, the insects gain bitterness in the body tissue. 23,24-Dihydrocucurbitacin D (Figure 5, MS:$M^+ - H_2O$, *m/z* 500) has been isolated from both species as a major substance causing bitterness (Nishida et al., 1990). The insects were extracted at least two days after compulsive feeding on *C. hilariana* roots. The yields of the compound present in the tissues were as high as 20 μg/insect in *C. arcuata* and 6 μg in *D. speciosa*. The major analogs (cucurbitacin B and D) seemed not to be sequestered in significant quantities in their body tissues. *D. speciosa* was also found to sequester hexanorcucurbitacin D (M^+, *m/z* 402) (Nishida et al. 1990). Although both 23,24-dihyrocucurbitacin D and hexanorcucurbitacin D are known from several curcurbit plants (Lavie and Glotter, 1971; Doskotch and Hufford, 1970), as well as from *C. hilariana* roots as relatively minor constituents (Nishida et al., 1990), it has not yet been demonstrated whether these chemicals are sequestered as a consequence of selective accumulation directly from their food or are metabolities of major cucurbitacins such as B and D.

Sequestration of Cucurbitacins by Asian Leaf Beetles. Similar to North American diabroticites, Asian pumpkin beetles in the genus *Aulacophora* are strongly associated with cucurbit plants. *A. foveicollis* is induced to feed by cucurbitacin E (Sinha and Krishna, 1969, 1970), and *A. coffeae*, *A. femoralis*, and *A. nigripennis* are also phagostimulated by cucurbitacin B and some other

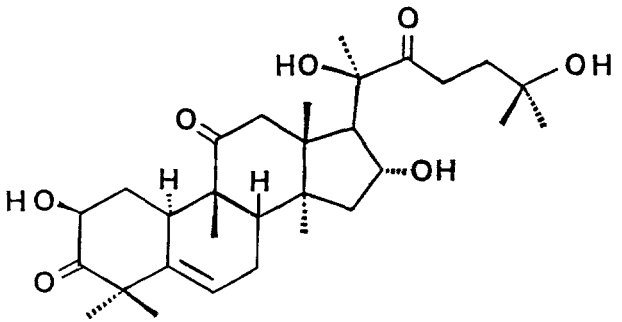

FIG. 5. Structure of 23,24-dihydrocucurbitacin D, a major cucurbitacin analog, sequestered by various chrysomelid leaf beetles associated with cucurbit plants.

analogs (Nishida et al., 1990). All four of these species store 23,24-dihydrocucurbitacin D in significant quantities in the body tissues (e.g., *A. coffeae*: 9 μg/insect; *A. foveicollis*, 3 μg/insect) (Nishida et al., 1990). Several analogs, including cucurbitacin B and D, were detected from overwintering *A. femoralis*, which had not fed at least a month. It is interesting that both the New and Old World leaf beetles are stimulated equally to feed by cucurbitacins (Metcalf, 1979, 1986) and selectively sequester the same compound, 23,24-dihydrocucurbitacin D, because many of the New World diabroticites are now polyphagous, while the Old World species remain oligophagous on Cucurbitaceae (Nishida et al, 1990).

ORIENTAL FRUIT FLY–METHYL EUGENOL ASSOCIATION

The Oriental fruit fly, *Dacus dorsalis*, is a polyphagous tephritid fly, infesting various kinds of fruit in the tropical and subtropical region. Methyl eugenol is known as an extremely potent kairomone for *D. dorsalis* males (Chambers, 1977). The insects are attracted to the chemical source and then feed on the intact chemical. *D. dorsalis* males visit various plants that contain methyl eugenol (Kawano et al., 1968; Shah and Patel, 1975; Fletcher et al., 1975). They probably ingest the essential oil components from the plant source. However, the biological significance of such specific attraction to methyl eugenol for the males has not yet been clarified. We have examined the volatile components in the male *D. dorsalis* body tissue and found that wild males possess varying quantities of two phenylpropanoid analogs, 2-allyl-4,5-dimethoxyphenol and coniferyl alcohol (Figure 6), while laboratory-reared males contain none of these components (Nishida et al., 1988a). *D. dorsalis* males

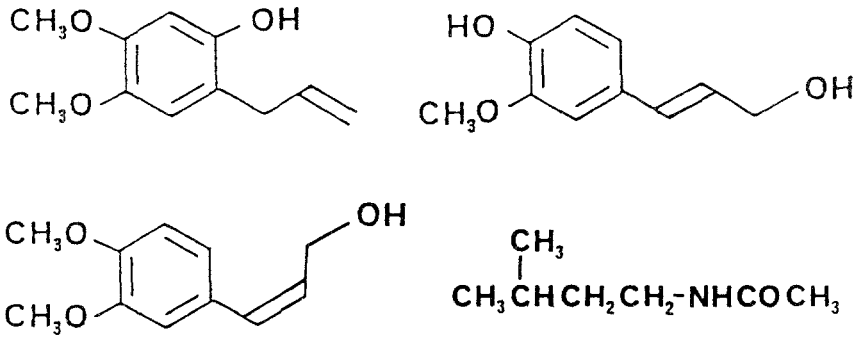

FIG. 6. Volatile components identified from rectal glands of *Dacus dorsalis* males. Upper left: 2-allyl-4,5-dimethoxyphenol. Upper right: coniferyl alcohol. Lower left: *cis*-3,4-dimethoxycinnamyl alcohol. Lower right: 3-methybutyl acetamide.

were shown to consume methyl eugenol in a short period and to convert this compound to these two components in their body tissue. Dissection of the male insect revealed that a large portion of the metabolites accumulated in the rectal glands, as much as 10 μg per gland. Males dissected within a few days after ingesting methyl eugenol also contained *cis*-3,4-dimethoxycinnamyl alcohol as a minor ingredient in the rectal glands (Figure 6). Besides these phenylpropanoids, 3-methylbutyl acetamide was also detected from the gland extracts of *D. dorsalis* males regardless of whether or not they fed on methyl eugenol (Nishida et al., 1988b). The gland complex of the male rectal sac has been suspected as the source of an olfactory pheromone (Kobayashi et al., 1978). *D. dorsalis* males produce a smokelike substance from the rectal glands, which has been shown to be attractive to virgin females (Ohinata et al., 1982). The volatiles from a chamber containing *D. dorsalis* males that had been fed methyl eugenol were collected on a short column of porapak-Q. The trap was replaced every 2 hr from noon until midnight. 2-Allyl-4,5-dimethoxyphenol was recovered from the aeration extract only during the time around sunset (1700–1900 hr), which coincides with the period when *D. dorsalis* males congregate and attract females (Figure 7). This evidence suggests a possible role of methyl eugenol as a pher-

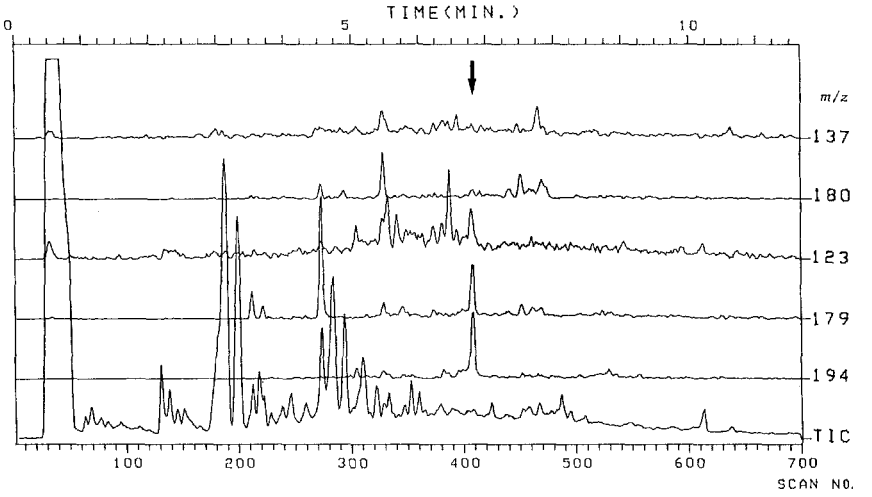

FIG. 7. Mass fragmentogram of an aeration extract in Porapak Q of *Dacus dorsalis* fed methyl eugenol around sunset (1700–1900 hr). This chromatography was done on a 23-m × 0.25-mm fused silica capillary coated with cross-linked-bonded methyl silicon (0V-101), 0.25 μm thick, programmed from 80°C (2 min holding) to 240°C at a rate of 10°C/min. The 70 eV spectra were used to plot *m/z* 194, 179, and 123 for 2-allyl-4,5-dimethoxyphenol, and *m/z* 180 and 137 for coniferyl alcohol. 2-Allyl-4,5-dimethoxyphenol is observed at a retention time of 6.8 min (scan number 409).

omone precursor in a courtship behavior, including male-to-male interaction. 2-Allyl-4,5-dimethoxyphenol also was found to attract male flies as potently as methyl eugenol in the field (Nishida et al., 1988a). Further behavioral study is needed to clarify the pheromonal function of the phenylpropanoids together with other ingredients found in the rectal secretions.

DETERRENT EFFECTS OF SEQUESTERED COMPOUNDS AGAINST PREDATORS

Preliminary observation suggested that *Athalia rosae* that had been fed with *Clerodendron* leaves taste extremely bitter and deter feeding of lizards (Johki and Nishida, unpublished data). The deterrent effect of clerodendrins to Japanese tree sparrows, *Passer montunus saturatus*, was tested as follows. Two square plates (16 × 16 cm) were placed next to each other in an outdoor bird-feeding arena in Kyoto City (Figure 8). Thirty rice grains treated with the test samples were placed in one plate, and 30 untreated grains were placed in the other plate. The activity was evaluated from the number of grains left from the initial 30 grains after exposure to sparrow feeding. Both clerodendrin B and D strongly deterred feeding of sparrows at 2 μg/grain (Table 1). Since the con-

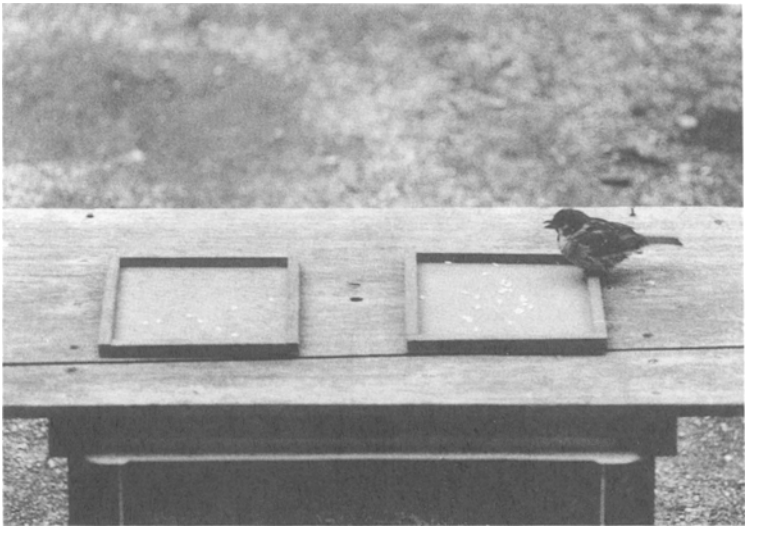

FIG. 8. Feeding deterrence bioassay of compounds sequestered by pharmacophagous insects against Japanese tree sparrows. Each tray contains 30 grains untreated (left) and treated with compounds (right). The number of grains remaining from the initial 30 grains was counted after exposure to sparrows.

TABLE 1. DETERRENT EFFECTS OF PLANT-DERIVED COMPOUNDS AGAINST SPARROWS IN RICE GRAIN FEEDING TEST

Sample	Effective dose (μg/grain)[a]
Clerodendrin B	2
Clerodendrin D	2
Cucurbitacin B	1
23,24-Dihydrocucurbitacin D	10
Hexanorcucurbitacin D	10
Methyl eugenol	10
2-Allyl-4,5-dimethoxyphenol	3
Coniferyl alcohol	>30

[a] Actual feeding counts and statistical comparisons for cucurbitacins and phenylpropanoids are given in Nishida et al., 1990 and 1988a, respectively.

centration of clerodendrins in female body tissue was found to be as high as 10 μg/insect and a portion appeared to be deposited on the cuticular surface, clerodendrins could effectively serve as a gustatory allomone.

The effective doses of cucurbitacin analogs sequestered by chrysomelid leaf beetles were determined similarly (Nishida et al., 1990). 23,24-Dihydrocucurbitacin, which is commonly sequestered by various kinds of beetles, was clearly rejected by sparrows. Cucurbitacin B, however, was shown to be the most deterrent, although it was detected only from *Aulacophora femoralis* in a relatively minor quantity (approximately 2 μg/insect).

In contrast to bitter clerodendrins and cucurbitacins, phenylpropanoid analogs found in the Oriental fruit fly do not taste bitter to humans. However, a significant deterrent activity against sparrows was observed for 2-allyl-4,5-dimethoxphenol at low levels. Conversion of methyl eugenol to more active oxidized forms might be adaptive in this insect. Strong rejection of methyl eugenol-fed *D. dorsalis* males by spiders and gekkoes also suggests a more general defensive role of the phenylpropanoids in the natural environment (K.H. Tan, personal communication).

DISCUSSION

Plants produce a wide variety of secondary metabolites to cope with various kinds of herbivorous attacks. Clerodane diterpenes have been shown to be potent antifeedands against several noctuid species (Kato et al., 1972; Kubo et al., 1976; Gueskens et al., 1983; Belles et al., 1985; Blarney et al., 1988), and

the triterpene cucurbitacins also act as deterrents against mites (DaCosta and Jones, 1971) and a chrysomelid beetle (Nielson et al., 1977). Some insects have not only overcome such chemical barriers after a long evolutionary history but also have developed the ability to sequester allomonal components originating from their specific host plants (Blum, 1981). In particular cases, a group of insects obtain nutrients from their host plants during larval stages and seek additional chemical substances from plants to protect themselves from predators during the adult stage. Here we have demonstrated three different cases of so-called pharmacophagous interactions. These insects are strongly attracted to the plant sources and gregariously engaged in feeding on the kairomones. Methyl eugenol is a potent attractant as well as a potent feeding stimulant for the Oriental fruit fly; however, clerodendrins and cucurbitacins are not likely to serve as airborne attractants because of their almost nonvolatile nature. They seem to act as feeding stimulants through the specific contact chemoreceptors located on the mouthparts of these insects (Metcalf, 1979). Pyrrolizidine alkaloids are also large molecules but apparently seem to attract a pharmacophagous moth species from distance (Boppré, 1986). The mechanisms of long-range attractions remain to be clarified.

It has been demonstrated that most of the chemicals sequestered by pharmacophagous insects are distasteful to sparrows. These "bitter" principles seem to act as defense substances against a wide variety of predatory animals, since some reptiles and spiders also rejected intact bodies of *Athalia rosae* fed with the *Clerodendron* plant and *Dacus dorsalis* fed with methyl eugenol. Ferguson and Metcalf (1985) have shown that the praying mantis, *Tenodera aridifolia sinensis*, rejects *Diabrotica* leaf beetles fed with bitter cucurbit fruits. However, some vertebrate predators obviously eat such bitter insects (Gould and Massay, 1984). Further work will be needed in order to know the "predatory spectrum" and allomonal mode of action of individual chemicals in their natural habitat.

The gregarious behavior of these pharmacophagous insects could be to effectively enhance their aposematic appearance against certain predators (Sillén-Tullberg, 1988) and could simultaneously serve as visual cues to congregate conspecifics. Three closely related turnip sawfly species, namely, *Athalia rosae*, *A. lugens infumata*, and *A. japonica* all look similar in that they each possesses orange-colored abdomens. All of these species are sympatric and feed together on *Clerodendron* leaves. They are regarded as Müllerian mimics. The plant seems to provide not only a chemical source, but also a rendezvous site for the sawflies, as mating behavior can be very frequently seen on the leaves (Kitano, 1988). Many cucurbitacin-associated chrysomelids also exhibit warning color patterns, some of the species resembling the color pattern of the turnip sawflies: black wings and a bright orange body. They also may be regarded as Müllerian mimics. Cucurbitacins and clerodendrins have been characterized as bitter principles in plants (David and Vallance 1955; Duncan et al., 1968; Lavie and

Glotter, 1971; Kato et al., 1971). In contrast, 2-allyl-4,5-dimethoxyphenol and coniferyl alcohol did not taste bitter to humans, although the former compound does repel sparrows. The male Oriental fruit flies seem to be chemically protected by foraging on methyl eugenol containing plants such as *Ocimum sanctum* (Labiatae), *Cassia fistula* (Leguminosae), and *Zieria smitii* (Rutaceae) (Shah and Patel, 1975; Kawano et al., 1968; Fletcher et al., 1975). The color pattern of the female fruit fly bodies is nearly the same as that of the male. It is postulated that females can be also protected if a large portion of the male population is unpalatable (automimicry, see Brower, 1969).

It has been suggested that the sequestered chemicals could trigger precopulatory behavior in the case of *Athalia rosae* and *Dacus dorsalis*. Similar relationships can be seen in danaid butterflies, in which the males utilize pyrrolizidine alkaloids as sex pheromone precursors (Boppré, 1978). Conner et al. (1981) suggested that hydroxydanaidal, accumulated in the coremata of a arctiid moth, *Utetheisa ornatorix*, might provide the means whereby the females assess the alkaloid content of the male and therefore his degree of chemical protection, in addition to its initial behavioral function as sex phermone. Males of the Oriental fruit moth, *Grapholitha molesta*, also have the ability to accumulate an additional quantity of one of the hairpencil pheromone components, ethyl *trans*-cinnamate, from their adult food, although it is uncertain whether the phenylpropanoid ester can serve as a defensive substance (Nishida et al., 1985). It would be of great interest to study further the pharmacophagous insects, because their apparently purposeless behavior might be explained by ecological adaptation mechanisms.

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LEPIDOPTERA AND PYRROLIZIDINE ALKALOIDS
Exemplification of Complexity in Chemical Ecology[1]

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Abstract—Pyrrolizidine alkaloids (PAs) are defensive secondary metabolites found in numerous plant groups. Various insects belonging to different orders have special requirements for these compounds and sequester them from such plants for their own defense and often as pheromone precursors. The fitness of these insects depends on PAs and, in some cases, PAs even act as regulators of androconial organ development. This article discusses selected behavioral, chemical, physiological, and phylogenetic aspects of insect–PA relationships, and raises questions about the complex interactions of the variety of PA-related adaptations as they occur among a diverse array of species. Although many superficial similarities are recognized, few generalizations can yet be drawn. However, insect–PA relationships not only exemplify basic features of chemical ecology but illustrate a multiplicity of aspects and adaptations, which we should expect to find in any thorough study of insect–plant relationship.

Key Words—Pyrrolizidine alkaloids, pharmacophagy, male pheromones, chemical defense, Lepidoptera, Danainae, Arctiidae, *Heliotropium, Senecio, Crotalaria*

INTRODUCTION

The large array of physiological, behavioral, chemical, and ecological phenomena involved in sequestration of secondary plant metabolites can, in practice, only be investigated in relatively few cases. Nonetheless, from this sample

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we hope to discover basic mechanisms—or even rules—governing chemically mediated interactions among organisms. The producers and consumers of pyrrolizidine alkaloids (PAs) appear to be well suited for such studies because they exhibit many features similar to those found in other systems of insect–plant relationships: on the one hand, PAs occur in a great variety of plant species and PAs are chemically distinct but quite diverse; on the other hand, various unrelated insects exhibit relationships to PA plants, and these are phenomenologically similar but nevertheless of different kinds. The polyphyletic development of both PA plants and the insect groups that exploit them enables us to undertake a comparative approach, leading to insights into evolutionary developments and processes. Furthermore, the associations of insects with PAs raise challenging questions for research into sensory physiology, sociobiology, biochemistry, and other fields peripheral to chemical ecology.

A significant and increasing amount of data on relationships between insects and plants containing pyrrolizidine alkaloids is now available. For example, we have learned that insects gather PAs not only from their primary host plants but also independently from nutrient uptake; insects store PAs for their protection against predators; and much is now known about PAs as precursors for male pheromones. Two examples suffice to illustrate the attraction of the subject: (1) Male milkweed butterflies (Danainae) visit withered PA-containing plants and extract PAs. These substances are required as precursors for biosynthesis of the quantitatively dominant pheromone component danaidone (Figure 1A) necessary for courtship success. In addition, males store PAs for their defense and also transfer significant amounts in the spermatophores, causing protection of

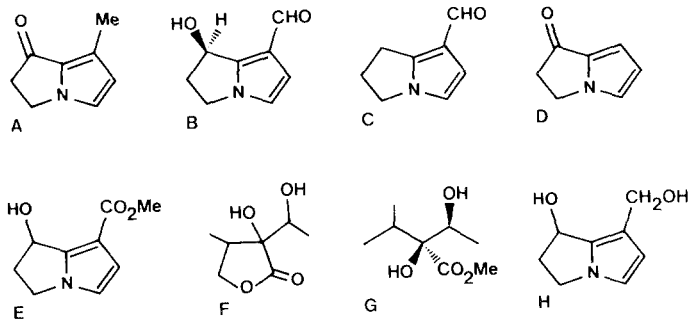

FIG. 1. Molecular structures of derivatives of pyrrolizidine alkaloids identified from androconial organs in Lepidoptera (see Table 4). A: danaidone, B: *R*-(−)-hydroxydanaidal, C: danaidal, D: nordanaidone (S. Schulz, personal communication), E: methylhydroxydanaidoate, F: ithomiine lactone [2-hydroxy-2-(1-hydroxyethyl)-3-methyl-γ-butyrolactone]; methylviridiflorate (G) and the necine pyrrole (H) are probably intermediates. G shows relative configuration.

females and eggs. (2) Larvae of *Creatonotos* (Arctiidae) are polyphagous but PAs alone can elicit feeding behavior. Those individuals that have PAs are protected from predators, and males with PAs also use them as precursors for biosynthesis of hydroxydanaidal (Figure 1B), which is emitted by coremata and appears to bring about the formation of mating assemblages. Thus there is an individual variation in the pheromonal strength of the males that is directly related to the amount of PAs gathered (as in the danaines, but here the amount per adult male is fixed for life); furthermore, in *Creatonotos* the development in size of the pheromone disseminating apparatus is also controlled by the amount of PA, i.e., PAs serve as organ-specific morphogens.

Neat as these brief summaries may sound, and although there is considerably more knowledge available than I have mentioned, our understanding of these phenomena is nonetheless superficial. Many aspects have yet to be investigated and, although many of the details involved might seem rather subtle, they are basic to our understanding not only of the two examples mentioned above, but also with respect to other organisms that sequester PAs.

This article addresses the multiplicity of phenomena involved in insect–PA relationships from a chemoecological perspective, and draws attention to some of the gaps in our knowledge that are rarely emphasized. Thus in the following sections, selected aspects are highlighted without comprehensive treatment of the subject and the respective literature. [For details and references on insects and PAs see reviews by Boppré (1986), Schneider (1987), Eisner and Meinwald (1987).]

PYRROLIZIDINE ALKALOIDS

PAs are chemically defined as ester alkaloids composed of an amino alcohol (necine; originating from 1-hydroxymethyl-pyrrolizidine) and mono- or dicarboxylic acids (necic acids; often C_7–C_{10} oxygenated and highly branched acids (Figure 2). Thus, saturated and unsaturated PAs are known that are mono- or diesters or macrocyclic diesters. PAs often occur as *N*-oxides; free-base alkaloids and their water-soluble *N*-oxides readily interconvert. To date, about 200 structures have been characterized, but many more have yet to be identified. [For details and references on PA chemistry see reviews by Bull et al. (1968), Robins (1982), Wróbel (1985), Mattocks (1986).]

PLANTS CONTAINING PAs

Plant species belonging to more than 60 genera in a dozen or so families have been recognized to produce PAs as secondary compounds. PAs have been found most widely in species of *Senecio* (Asteraceae), *Heliotropium* (Boragi-

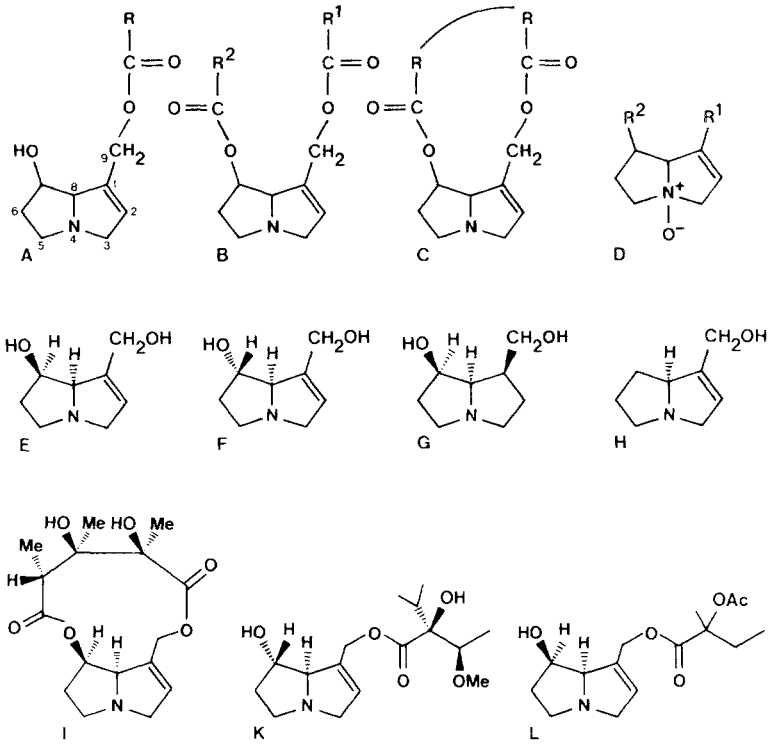

FIG. 2. General structure of pyrrolizidine alkaloids (A–C) and PA *N*-oxides (D) and molecular structures for examples of necine alcohols (E: retronecine; F: heliotridine; G: platynecine; H: supinidine) and of free bases (I: monocrotaline; K: heliotrine); L: the insect metabolite callimorphine. E–L show absolute configurations.

naceae), and *Crotalaria* (Fabaceae) (Smith and Culvenor, 1980), but these genera have been investigated much more thoroughly than others because of their importance for domestic animals and man. Their PAs usually consist of the necines retronecine and heliotridine (Figure 2E,F); retronecine appears as the most common amino alcohol in PAs.

Generally, a given plant species contains several PAs; such a set consists of PAs of similar structure, and these PAs tend to be characteristic of plant genera or even some higher groupings. Reported amounts of PAs are >5% (maximum: 17.99% dry weight in *Senecio riddellii*; Molyneux and Johnson, 1984) or as low as 0.001% of dry weight; a common figure is 0.1% or so. Considerable intraspecific variation also occurs (e.g., Johnson and Molyneux, 1985), involving both qualitative and quantitative composition as well as spatial

and temporal distribution of PAs, a result of the physiological state of the individual plant as well as of its environmental conditions.

Although we talk often about PA plants, from a chemoecological point of view they are anything but uniform. The (chemical) definition of PAs is quite broad, and our biological knowledge should forbid us to group all the different structures under one term; for convenience only, the general term is still employed. Provoked by the pathogenicity of (certain) PAs, an impressive amount of knowledge on PA chemistry and toxicology has been gathered, but ecological aspects have been relatively neglected. Most studies have focused solely on the identification of the molecular structures; thus for extraction and isolation of PAs standard techniques have been employed, which, on the one hand, usually involve dried plant material only and, on the other hand, carry the risk of chemical degradation (e.g., reduction of *N*-oxides; cf. Hartmann and Toppel, 1987) during the procedures.

Some valuable information is available on ecophysiological matters, but it is presently incomplete and too limited to permit an understanding of PA plants in a wide sense. Evidence from *Senecio* and *Crotalaria* favors the idea that *N*-oxides are the form by which PAs are synthesized, translocated, and accumulated, while seeds seem to contain free-base alkaloids exclusively (cf. Hartmann et al., 1988). Is this situation common to the entire spectrum of PA plants? Studies involving examples from a range of unrelated species and considering different physiological states are urgently required, since the physicochemical properties of free bases and *N*-oxides are quite different and must have different biological effects on insects in the context of perception, toxicity, degradation, solubility, and transportation. Further basic tasks include details on the occurrence of PAs in nectar, the changes in PAs when a plant is withering or drying, and the stability of PAs as well as their derivatives (see below).

PAs IN PLANTS—WHAT FOR?

The majority of known PAs cause serious diseases in domestic animals and man—the leading motive for most studies on PAs. In contrast to many other secondary plant metabolites, the noxious actions of PAs are not instantaneous but appear only after weeks or months of incubation and in an indirect way. In the vertebrate metabolism, unsaturated ester alkaloids (those with 1,2-dehydro-1-hydroxymethyl-pyrrolizidines) are converted into pyrroles (Figure 1H), which are responsible for hepatotoxic, mutagenic, oncogenic, and other deleterious effects. [For review and references on toxicological aspects see Bull et al. (1968), Mattocks (1986).] If PAs with other structures are harmless, what are their biological roles?

Although some domestic herbivores consume PA plants (particularly if there is shortage of other food), PAs actually protect their producers significantly from herbivory. Grazing animals usually avoid PA plants, apparently because of their deterrent taste; for humans, PAs taste bitter. One can speculate that it has to be a kind of gustatory "aversion receptor" that protects herbivores from uptake of PAs, i.e., from being poisoned. This receptor probably has low specificity, otherwise the great number of different PAs would not be explicable since one would have to expect selective pressure on the plants to synthesize PAs with less diverse structures. Is it a general "bitter receptor," with a wide reaction spectrum, i.e., does it sense other types of noxious compounds, too? If so, it might not have evolved as a response to the "invention" of PAs by plants but antedate this incident(s). Could we then propose that the (PA) plants have merely taken advantage of a sensory system generally present in animals? (See below on aversion in nonherbivorous vertebrates and insects.) Unfortunately, so far, this matter represents nothing but speculation.

To a great extent, PAs appear to protect plants from damage not only by vertebrates but also by insects. Comparatively few species are known to utilize PA plants as hosts, and the aversion receptor postulated above might be present in insects also: PA-contaminated but otherwise adequate food is rejected by locusts, many lepidopteran larvae, cockroaches, ants (Bernays and Chapman, 1977; Bentley et al., 1984; Boppré et al., in preparation)—even by species that naturally do not encounter PAs (e.g., *Periplaneta, Blatta*). PA-containing nectar is avoided by many nectar-foraging species, and insects storing PAs are protected from predators including invertebrates (see below). It must be emphasized, however, that we have no idea what would happen to an unadapted insect if it lacked the taste barrier and took up PAs. One cannot predict the physiological reactions of invertebrates from knowledge of the toxicology in vertebrate metabolism.

The undoubted protective role that PAs have for plants cannot explain the entire phenomenology of such compounds within the plant kingdom. Why do we find amounts of PAs that are much higher than required for deterring herbivores? For example, seeds of *Crotalaria* may contain as much as 5% PAs by dry weight. Of course, the reproductive organs may require the best protection possible, but the plant should not invest more energy in PA synthesis than needed. Toppel et al. (1988) have recently shown for *Crotalaria* that in the course of germination PAs are metabolized as a nitrogen source; the seedlings do not contain PAs at all. Thus, the secondary compounds are also relevant for primary metabolism, a fact that also can be inferred from the great variations observed in PA content of the plants (see below) but which by no means contradicts the role of PAs as defensive chemicals.

PA-ADAPTED INSECTS

Insects have selectively adapted to most of the defensive mechanisms of plants; a variety of species utilize PA plants as hosts, and yet others depend on PAs and gather them from plants independently of feeding. Not very many species have been investigated in detail. The following accounts focus on Lepidoptera and are further restricted to examples dealing with the Asteraceae, Boraginaceae, and Fabaceae, simply because other systems have hardly been looked at.

Considering the disjunct phylogeny of taxa making use of PA plants and the different types of PA insects, adaptations to utilize PAs must be polyphyletic in origin. As with PA chemistry, few other generalizations can be drawn, although the phenomena obviously exhibit similarities. In any case, one has to differentiate between three types of associations (Table 1): insects taking advantage of (1) the nutritional value only, (2) both the primary and the secondary metabolites, and (3) PAs alone.

Insects utilizing PA plants solely as food sources demonstrate, perhaps, the most original adaptation, apparently enabling them to exploit a food resource with limited competition. We know too little of the few species belonging to this category, but it seems that they are mostly polyphagous and handle PAs unspecifically by converting and/or excreting them.

Lepidoptera restricted to PA-containing host plants (e.g., *Tyria, Utetheisa, Nyctemera*) seem to take advantage of PAs themselves in one way or another. They have probably adapted to avoid harm and, apparently, they have means of recognizing PA plants. Since a PA plant is not only characterized by the possession of PAs, one can imagine a variety of cues that are capable of guiding an insect to its host, but critical investigations regarding this point are entirely lacking. Host records of insects using PA plants indicate, however, that different Lepidoptera do use different stimulus patterns for egg-laying and/or phagostimulation, because various species exhibit different host ranges (Table 2), indicating that PAs cannot be the only cues (but see discussion of pharmacophagy below).

Straightforward detection and recognition of PAs is a basic feature of pharmacophagous species. By definition, species of this category search for the allelochemics directly, take them up, and utilize them for a specific purpose other than primary metabolism or (merely) host recognition (Boppré, 1984b), i.e., they often gather secondary plant compounds independently of foraging for nutrients. Pharmacophagy with respect to PAs has been demonstrated for *Creatonotos* larvae (Table 2) but is most easily recognized in all those Lepidoptera (and other insects) that, in their adult stage, visit withered or decaying PA plants

TABLE 1. EXAMPLES OF LEPIDOPTERA AND OTHER INSECTS SHOWING DIFFERENT KINDS OF PA SEQUESTRATION

ORDER, Family, *Genus*	Larval host(s) contain PAs	Adults visit dry PA plants		Adults store PA	PA-derived pheromone[b]
LEPIDOPTERA					
Noctuidae					
Spodoptera[1]	− / +	−		−	−
Arctiidae					
Spilosoma[1]	− / +	−		− / +	−
Arctia[1]	− / +	−		− / +	−
Creatonotos[1]	− / +[c]	−		− / +	− / +
Phragmatobia[3]	− / +	−			+
Pyrrharctia[3]	− / +	−			+
Tyria[1,4]	+	−		+	−
Amphicallia[1]	+[c]	−		+	−
Utetheisa[5]	+[c]	−		+	+
Nyctemera[6,7]	+[c]	males	females	+	− / ?
Rhodogastria[7]	−	males	females	+	−
Halysidota[8–10]	−	males	females		
Cisseps[8–10]	−	males	(females)		+
Ctenuchiidae					
Euchromia[7]	−	males	females	+	−
Ctenucha[10]	−	(males)	females		
Lymire[8,9]	−	males	(females)		
Euceron[8,9]	−	males	(females)		
Danainae[11]					
Danaus	−	males		+	+
Amauris	−	males		+	+
Euploea	−	males		+	+
Ithomiinae[8,12]					
Several genera	−	males		+	+
COLEOPTERA					
Chrysomelidae					
Longitarsus[13]	+		−		
Gabonia[14]		males			
Nzerekorena[14]		males	females		
DIPTERA					
Melanochaeta[15]		males	females		
Eutropha[15]		males	females		
Chlorops[15]		males	females		

TABLE 1. Continued

ORDER, Family, *Genus*	Larval host(s) contain PAs	Adults visit dry PA plants	Adults store PA	PA-derived pheromone[b]
ORTHOPTERA				
Pyrgomorphidae				
Melanoplus[16]	− / +	−	−	−
Zonocerus[17]	− / +	males females	+ / −	

[a]Note that the list is not comprehensive and, in part, generalized; for simplification it is based on genera, which does not necessarily imply that the information indicated is valid for all species (e.g., of *Halysidota longa* 99% males are attracted to PA baits, of *H. tessellaris* 90.9% females; Goss, 1979); the levels of investigation are also quite different. The references given provide a basis to the partly extensive literature. [1]Rothschild et al. (1979); [2]Boppré and Schneider (1989); [3]Krasnoff et al. (1987); [4]Ehmke et al. (personal communication); [5]Culvenor and Edgar (1972), Conner et al. (1981); [6]Benn et al. (1979); [7]Boppré (1981); [8]Pliske (1975); [9]Goss (1979); [10]Krasnoff and Dussourd (1989); [11]refs in Ackery & Vane-Wright (1984); [12]Brown (1984, 1987); [13]Boppré (1983); [14]Scherer and Boppré (1990); [15]Boppré and Pitkin (1988); [16]Ehmke et al. (1989); [17]Bernays et al. (1977), Boppré et al. (1984).

[b]See Table 4.

[c]See Table 2.

(Table 1) and extract PAs from the dry material by using a liquid released through their proboscides. In most of these species the larvae feed on PA-free hosts, but this need not necessarily be the case (see *Nyctemera*, Table 1). In experimental situations, they will also go for certain pure PAs, i.e., orientation to PA sources has to be mediated olfactorily and also be solely related to PAs and not to any other plant product.

We do not know much about preferred species of PA plants, but plants with structurally different PAs are attractive (Table 3). Because of their high molecular weight, intact esters are unlikely to have sufficient volatility to mediate attraction. But we must ask precisely what cue(s) enable PA pharmacophagous insects to recognize PA sources. Recently, two independent approaches to this question revealed that hydroxydanaidal (Figure 1B), previously well known as male pheromone (see below), is the airborne signal from a variety of PA sources (Krasnoff and Dussourd, 1989; Bogner and Boppré, 1989; compare Pliske et al., 1976). Discrepancies exist, however, since Krasnoff and Dussourd (1989) found *S*-(+)-hydroxydanaidal more attractive than the respective *R*-(−)-enantiomer, while Bogner and Boppré (1989) found the opposite. The latter authors put forward evidence that hydroxydanaidal is a spontaneous degradation product that emanates in minute quantities from all those PAs that have either retro-

TABLE 2. EXAMPLES OF ARCTIID MOTHS ASSOCIATED WITH PA PLANTS DEMONSTRATING HOST RECOGNITION/SELECTION IS NOT ONLY DUE TO PAs[a]

	Amphicallia	*Nyctemera*	*Utetheisa*	*Creatonotos*
Heliotropium	−	−	+	+
Crotalaria	+	−	+	+
Gynura	−	+	−	+
Non-PA plants	−	−	−	+
Purified PAs	?	?	−	+

[a]Boppré, unpublished field records from Kenya and laboratory tests.

necine or heliotridine as the necine and are present as free bases. This finding explains the given but not very high specificity of attraction and suggests why plants must be withered (or damaged) to be attractive and lure best if remoistened. The study also demonstrated receptor cells for the perception of secondary plant metabolites that have similar specificity and sensitivity as receptors for sensing female pheromones in moths (e.g., Mustaparta, 1984). (At close range to the source, gustation also seems to be involved: Lepidoptera probe with their proboscides before eventually applying liquid to extract and imbibe PAs.)

Field observations on the biology of Lepidoptera that gather PAs as adults have revealed that nectar can be a potential source of PAs. Chemically, PAs have been recognized in honey of *Senecio* and *Echium* (up to 3.9 ppm; Deinzer et al., 1977; Culvenor et al., 1981), and flowers of the Eupatoriae (Compositae) are considered to be a major source of PAs for Lepidoptera (e.g., Brown, 1984). The flowers of the PA plant *Gynura scandens* are exclusively visited by PA insects, and these exhibit the same sex bias as at withered plants or artificial baits; other Lepidoptera completely ignore these flowers (Boppré, unpublished). Some novel PAs were first found in *Euchromia* moths that had visited *Gynura* flowers (Boppré and Edgar, unpublished; compare Wiedenfeld, 1982). In contrast, flowers of other PA plants (e.g., *Heliotropium*) are utilized by a wide spectrum of nectar foraging insects. This suggests that the nectar of PA

TABLE 3. PLANT GENERA MOST FREQUENTLY USED AS PA SOURCES BY ADULT LEPIDOPTERA

Asteraceae	*Ageratum, Eupatorium, Senecio, Gynura*
Boraginaceae	*Heliotropium, Tournefortia*
Fabaceae	*Crotalaria*

plants does not necessarily contain these secondary products—an aspect requiring further investigation. Furthermore, some orchids (*Epidendrum* sp.) are pollinated exclusively by adult Lepidoptera that are pharmacophagous with respect to PAs; apparently, these plants do not provide PAs but cheat PA insects to take advantage of specialists for pollination (Goss and Adams, 1976; DeVries and Stiles, 1989).

How elaborate the gathering of PAs can be is shown by danaine butterflies that vigorously scratch plant tissue with their legs to get access to PAs (which they then suck up with their proboscides). They need, however, a starting point such as a hole made by beetles (Boppré, 1983); undamaged tissue is ignored, apparently lacking the volatile cue for attraction.

Foraging PAs can thus be of great cost with respect both to phylogenetic adaptations and to the time and effort invested by individuals. One may therefore expect that PAs play significant roles in the lives of PA insects.

PAs AND THEIR ROLES FOR INSECTS

PAs for Defense. The great majority of insects that utilize PA plants in one or another way are aposematically colored and behave conspicuously. This suggests, in combination with the information above on the protective role of PAs for plants, that insects do store PAs and use them for their own protection. Storage of PAs by Lepidoptera was first demonstrated by Aplin and Rothschild (1968, 1972), and numerous similar studies have been undertaken since; the analyses reported by Brown (1984a,b, 1987) are outstanding for their detail.

Most species related to PA plants that have been analyzed chemically have been found to store PAs in their bodies (Table 1); the amounts can be as high as 20% of the insect's dry weight (e.g., Brown, 1987). Detailed analyses on the qualitative and quantitative composition of PAs in insects reveal, however, many intra- and interspecific differences. PAs found in insects do not necessarily represent fingerprints of the PAs found in their host(s); rather, there are great differences in the patterns, both qualitative and quantitative (e.g., Culvenor and Edgar, 1972; Aplin and Rothschild, 1972); even sex differences occur in PA storage by larvae that had fed on the very same individual plant. Are we dealing with selective uptake, selective metabolism, or perhaps selective conversion due to selective use? What is the role of "callimorphine" (Figure 2L), a PA not known from plant sources but found in several arctiids jointly with host plant PAs (Aplin et al., 1968; Edgar et al., 1980; Wink et al., 1988; L'Empereur et al., 1989). Unfortunately, studies considering both plants and insects at the same time are rare. We also need many more qualitative and quantitative PA profiles of several insect species to learn about individual (and sex-specific) variation, selectivity of uptake and utilization in sympatric species, and differences among populations. Further questions of general importance include: do

adult Lepidoptera extracting PAs from withered material of a given plant obtain the same amounts and types of PAs as obtained by larvae feeding on the living tissues? Extracts of insects usually reveal a high percentage of PAs in their *N*-oxide forms; did the insects take up *N*-oxides or did they oxidize free bases? If the latter (as demonstrated for *Rhodogastria*; Wiedenfeld and Boppré, unpublished) is the case, is *N*-oxidation caused by an adapted mechanism or is it simply an obligatory metabolic process? Are the polar *N*-oxides more or less or equally defensive (and/or repellent) compared to free bases or are they better suited for transport within the body? Thus, merely recognizing that an insect is associated with a PA plant is insufficient to predict if and how it sequesters the plant chemicals.

Although storage of PAs does not inevitably imply a protective function, we know from the meticulous studies of Eisner (1980, 1982) and Brown (1984a,b, 1987) that Lepidoptera (*Utethesia* and Ithomiinae) can be protected from predation (by *Nephila* spiders) by stored PAs they had gathered, respectively, as larvae or adults. Both these workers also demonstrated that, in their examples, PAs alone were responsible for protection.

With several other species the situation is more complex since they possess self-manufactured, toxic secretions in addition to PAs (see Blum, 1981; Rothschild, 1985; for general accounts and references) and/or they store other chemicals sequestered from plants. For example, *Danaus plexippus* can store cardenolides from larval hosts and the adult butterflies gather PAs in a pharmacophagous way (e.g., Brower, 1969; Kelley et al., 1987);[2] however, Ithomiinae and *Euploea core*, for example, do not store the defensive compounds present in their host plants (Brown, 1984a,b, 1987; Malcolm and Rothschild, 1983) and seem to rely only on PAs obtained as adults. Are different (sets of) compounds effective against different predators, do they act synergistically, and/or are several chemicals utilized to ensure protection if one or the other type is not available at a given habitat or time? Last, but not least, the considerable intraspecific variation in PA storage, particularly in pharmacophagous species, and the resulting "palatability spectrum" is of great relevance and deserves detailed study—also in the context of (auto-)mimicry.

At this point we should consider the potential predators for PA insects. Since PA insects are diverse with respect to, among other factors, size, activity pattern, and habitat, the range of potential predators is great and includes organisms with quite different physiology and life-styles (vertebrates as well as invertebrates). As with plants (see above) the question of unpalatability vs. toxicity arises (see also discussion by Brower, 1984). Is the knowledge of the

[2] Interestingly, often (always?) the ability to utilize PAs is associated with the ability to utilize cardenolides, but no common denominator of PAs and these glycosides is recognized (e.g., Rothschild et al., 1970).

toxicology, indicated above and largely derived from studies of domestic animals, really applicable to insectivores such as birds, bats, lizards, and toads? Would, for example, amounts of PAs accumulated through insectivory fall below the poisoning threshold? Likewise, in this context it must be asked again: why do insects usually avoid PAs and what kind of harm do they risk with ingestion of these compounds?

Taste rejection of PA-storing insects by predators suggests that PAs are located on the prey's outer surface. Indeed, biological observations verify this: *Nephila* spiders release Ithomiinae and *Utetheisa* unharmed from their webs (Eisner, 1982; Brown, 1984a), and *Creatonotos* and *Rhodogastria* survive attacks by toads and lizards, which spit them out (Boppré, unpublished). From Brown's studies we know about the quantitative distribution of PAs/PA *N*-oxides in the various body parts of ithomiine butterflies (accumulation in exoskeleton and reproductive tissues), but we lack information as to how PAs are transported. If PAs are ingested by larvae, the problem of how PAs reach the cuticular surface is less intriguing than in those Lepidoptera that gather PAs as adults, i.e., after the cuticle has been formed. In many arctiid adults, malodorous froth exuded from prothoratic glands immediately after an attack seems to support instant taste recognition. If adults have access to PAs, the froth of *Rhodogastria* is loaded with PA *N*-oxides (Wiedenfeld and Boppré, unpublished).

The transport of PAs in insect bodies must relate to how PAs pass the gut membranes. Because PAs are large molecules, some kind of carrier molecules should be expected (Hartmann, 1985; Wink and Schneider, 1988). Surprisingly, experiments with *Creatonotos*, the larvae of which happily consume glass-fiber disks impregnated with PAs (Boppré, unpublished), indicate that if the insects had ingested pure PAs in this way the effect of these chemicals on morphogenesis (see below) is 10–100 times weaker than if PAs had been provided naturally, i.e., unextracted via plant tissue (Boppré, unpublished). This may suggest attachment of PAs in plants to other molecules, resulting in more efficient metabolism (translocation, storage, conversion?) in insects. Perhaps, we even should ask whether poisoning of vertebrates is greater if PAs are ingested with plant tissue then in pure form, implying that LD_{50} tests with pure chemicals might be misleading. Based on available evidence, storage for defense seems to be the primary function in most, if not all, insect–PA relationships, but in many it is not the only one.

PA-Derived Male Pheromones. In Lepidoptera, utilization of PAs as precursors for male pheromones is widespread although not general (Table 1). Figure 1 shows the structures of PA-derived compounds identified from androconial organs in Lepidoptera, and Table 4 links them to the respective genera. It shows that unrelated species end up with similar or even identical pheromone components, regardless of the stage in which PAs are consumed. Nevertheless, in chemoecological terms this is not a basis for establishing a rule. As the infor-

TABLE 4. LEPIDOPTERA USING PA-DERIVED MALE PHEROMONES[a]

Family genus	A	B	C	D	F	Amount (μg/male)	Release
Danainae[1,2b]							
Danaus	x					0–500	I
Lycorea	x						I
Amauris	x	(x)					I
Euploea		x	(x)				I, II
Ithomiinae[3–5b]							III
Prittwitzia				x	x	0.01–0.2	
Mechanitis					x		
Tithorea					x		
Arctiidae[c]							
Utetheisa[6]		x	x			0–30	I
Phragmatobia[7]		(x)	x			0–24	I
Pyrrharctia[7]		x	(x)			0–10	I
Cisseps[8]		x				0–0.12	I
Creatonotos[9]		x				0–400	III

[a] A: danaidone, B: hydroxydanaidal, C: danaidal, D: methyl-hydroxydanaidoate, F: ithomiine lactone, (Figure 1), the amounts found, and the behavioral situation for release [I: briefly, close to the female; II: patrolling with expanded hairpencils; III: for long, stationary (luring males and females?]. [1]Refs. in Ackery and Vane-Wright (1984); [2]Schulz (1987); [3]Edgar et al. (1976); [4]Haber (1978); [5]Schulz et al. (1988); [6]Culvenor and Edgar (1972), Conner et al. (1981, 1989); [7]Krasnoff et al. (1987), Krasnoff and Roelofs (1989); [8]Krasnoff and Dussourd (1989); [9]Schneider et al. (1982); Wunderer et al. (1986); Boppré and Schneider (1989).

[b] PA uptake by adults.

[c] PA-uptake by larvae.

mation provided in Table 4 also indicates, the respective species differ strikingly in the quantities of PA-derived compounds, they have quite different courtship strategies, and their pheromones come into play in different behavioral contexts, suggesting different roles, i.e., broadcasting different messages to the female (Boppré, 1984a).

In *Danaus chrysippus* (and probably many other danaine butterflies), mechanical contacts between abdominal hairpencils and alar glands are required for synthesis of danaidone (Boppré et al., 1978). Thus, in addition to the costs of foraging for PAs (plus producing appropriate enzymes), the males employ two sets of glandular organs and perform a peculiar precourtship activity. Must danaidone synthesis occur outside the body in order to avoid poisoning by PA metabolites? Perhaps so, but other species (e.g., *Lycorea*) manage without alar organs. It is also puzzling why so many species utilize hydroxydanaidal as a male pheromone (component). The idea that this molecule might be less costly to synthesize than other PA derivatives (e.g., danaidone) receives some support

by finding that hydroxydanaidal appears as a spontaneous degradation product (see above).

There are two enantiomeric forms of hydroxydanaidal but the androconia of *Creatonotos* and (all?) other Lepidoptera that use PAs as precursors emit *R*-(−)-hydroxydanaidal only (Figure 1B, Table 4). This detail is by no means trivial in an ecological context because insect olfactory receptors usually discriminate between enantiomers and, according to textbook knowledge, the enantiomeric configuration should not be changed in the course of biosynthesis of hydroxydanaidal from PAs. It was completely unexpected to find, from feeding experiments, that *Creatonotos* can utilize PAs with retronecine or heliotridine, in the latter case converting the configuration at C-7 (Bell et al., 1984; Bell and Meinwald, 1986; compare Wink et al., 1988). This mechanism, in consequence, enables *Creatonotos* to take advantage of a wider spectrum of species of PA plants than it could otherwise do. What happens in such insects as *Euploea*, which gather PAs as adults?

Because hydroxydanaidal is not only a pheromone but also the volatile principle responsible for attraction of insects to PAs (see above), certain insects might get confused—PA sources smell like male androconia and, in turn, androconia smell like PA sources. In fact, coremata of *Pyrrharctia* and *Creatonotos* attract PA insects (Krasnoff and Dussourd, 1989; Boppré, unpublished).

Referring to the androconial secretions as male pheromones is not very meaningful because the messages male pheromones carry are generally little understood and certainly not uniform (Boppré, 1984a). In any case, it is very surprising that a diversity of Lepidoptera use derivatives of (defensive) plant metabolites as male pheromone precursors. Utilization of plant products for pheromone biosynthesis is not restricted to PAs; the bark beetles (Scolytidae) provide a well-known example (e.g., Borden, 1985). However, in certain PA insects the question has a special dimension because of the linkage between chemical defense and chemical communication.

Based on the fact that hydroxydanaidal-deficient males of *Utetheisa* are unsuccessful in courtship, Conner and Eisner suggested the females might sexually select for males with a high ability to sequester PAs, a trait that might be inheritable (Eisner, 1980; Conner et al., 1981). Finding that males also donate substantial amounts of PAs with their spermatophores (Brown, 1984, 1987) resulted in an even more plausible hypothesis: since the male pheromone is derived from the nuptial gift, it might indicate to the female the amount of protective chemicals she can expect to gain during copulation (Eisner and Meinwald, 1987; Dussourd et al., 1988). Although several of the assumptions lack confirmation as yet, for *Utetheisa* there is some good evidence in favor of the hypothesis (see also Conner et al., 1989). The hypothesis is also of great value for studies on other species utilizing PAs for both defense and chemical communication. However, considering the differences in courtship behavior, the

fact that some species have to make do with a fixed amount of PAs gathered as larvae while others can increase their drug budgets as adults, and considering the existence of additional chemicals (e.g., extracts of male danaine hairpencils revealed bouquets comprised of up to 50 volatiles found only in the androconia; Schulz, 1987), without modification the hypothesis is unlikely to be equally valid for the entire variety of species.

More thorough studies focusing on how male butterflies handle ingested PAs are required for our understanding of the chemoecological aspects. What is the order of priority and what are the budgets if PAs are used for pheromone biosynthesis, for protection, and for incorporation into spermatophores? This question is of particular importance if PA resources are limited, and the answer must be expected to differ among species of PA insects. Furthermore, are the various PAs obtained by an insect from different plant species used equally or are there differences such that, for example, only certain PAs are stored for defense while others are converted into pheromones? Is there a difference between utilization of *N*-oxides and free bases, and/or are pure PAs less efficiently used (see above)?

Further questions arise from sex-specific sequestration of PAs. Among those species gathering PAs as adults, there is often a male bias, but others have female and yet others no sexual bias (Table 1). In *Creatonotos*, there is a significant sexual difference in the amount of PAs taken by larvae in ad libidum experiments (Boppré, unpublished). Particularly informative might be comparative studies with closely related species that behave differently. For example, *Amauris ochlea* and *A. niavius* are sympatric congeners. In the former, males exclusively are found at PA baits or plants, but in the latter species, a high proportion of females also visit such PA sources (Boppré, unpublished). Again a sensory physiological aspect appears, which seems promising for a study on the evolution of receptor specificity.

PAs as Morphogens. A unique effect of PAs has been recognized in arctiid moths of the genus *Creatonotos*. The amount of PAs consumed by larvae not only determines the amount of male pheromone [*R*-(−)-hydroxydanaidal] but also the size of the coremata (Schneider et al., 1982; Boppré and Schneider, 1985, 1989). Morphogenesis of these androconial organs (and of these alone) is quantitatively dependent upon the amount of PAs ingested by a larva; there is no previous knowledge of a similar morphogenic effect generated by a secondary plant metabolite. Recently, however, we found considerable variation in the size of coremata in a field population of *Teracotona* (Boppré, unpublished), and we are curious to study if PAs (or other secondary plant substances) act as morphogens.

Other Functions? There are many further facets in insect–PA relationships, making the complex situation discussed above even more involved, but the subject cannot be treated comprehensively here. One could add that Pliske

(1975a,b) and Goss (1979) speculate on a nutritive function for PAs, but there is no evidence for such an idea. In the case of Danainae and Ithomiinae there is debate on the evolutionary origins of their relation to PA plants. However, the most significant increase in the complexity of the subject occurs when non-lepidopteran insects associated with PA plants are considered in addition; Table 1 lists just a few examples.

EPILOGUE

PAs belong to the best investigated natural products and many aspects of sequestration of these allelochemics by insects (mainly Lepidoptera) have been studied. Despite our present knowledge, many questions naturally arise that demonstrate we have seen only "the tip of the iceberg" so far. The many aspects described here are of unequal importance and cannot be considered at once; they are neither meant to cause confusion nor to discourage specific studies; rather, they are intended to stress the research challenge provided by insects and PAs. However, we have to consider the complexity which is, I think, the fascination of chemical ecology. We should increase detailed studies and stress comparative approaches but refrain from generalizing conclusions; as shown above, to date we know quite a few examples but there is no model representative for insect–PA relationships.

Raising open questions on insect–PA relationships should also illuminate the many tasks for biological disciplines not directly involved in chemical ecology. These include studying such different subjects as receptors, morphology, ethology, orientation, phylogenetics, and even developmental processes.

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IRIDOID GLYCOSIDE CONTENT OF *Euphydryas anicia* (LEPIDOPTERA: NYMPHALIDAE) AND ITS MAJOR HOSTPLANT, *Besseya plantaginea* (SCROPHULARIACEAE), AT A HIGH PLAINS COLORADO SITE[1]

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Abstract—The checkerspot butterfly, *Euphydryas anicia*, utilizes mainly *Besseya plantaginea* and only occasionally *Castilleja integra* as a larval hostplant at Michigan Hill, a few kilometers from a site where *C. integra* is used by over 90% of the butterflies. The *B. plantaginea* leaves that are consumed contain 9–22% iridoid glycosides, composed mainly of catalpol and catalpol esters, while larvae from the same plants contain 6–18% iridoids, mainly catalpol and no esters. Field-collected adult butterflies contain 0.5–4.3% iridoids. Laboratory-reared adults secrete iridoids in the meconium upon eclosion and retain similar amounts. The adult and meconium iridoid content is considerably lower than in the larvae, and metabolism in the pupal stage may be occurring.

Key Words—*Euphydryas anicia*, Lepidoptera, Nymphalidae, *Besseya plantaginea*, Scrophulariaceae, iridoid glycosides, sequestration, plant-insect interactions, herbivory.

INTRODUCTION

We recently reported (Gardner and Stermitz, 1988) the first detailed study of iridoid glycoside sequestration patterns in a natural lepidopteran population: *Euphydryas anicia* (Nymphalidae) hosted by *Castilleja integra* and *Besseya*

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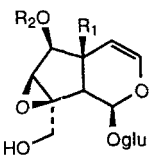
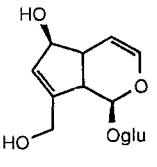
plantaginea (Scrophulariaceae) at a high plains Colorado site (Red Hill). A second localized population of the same checkerspot butterfly occurs on Michigan Hill, about 8 miles (13 km) east of Red Hill. Both host-plant species are also present at Michigan Hill. Preliminary analysis of field-caught adult butterflies from Michigan Hill showed that they contained the iridoid glycoside catapol, **1**, but lacked macfadienoside, **2**, an iridoid exclusive to *C. integra*, which was present in over 90% of the butterflies at Red Hill (Gardner and Stermitz, 1988) (Figure 1). Both host-plant species contain catapol and *B. plantaginea* contains catalpol esters that are hydrolyzed by the larvae to catalpol.

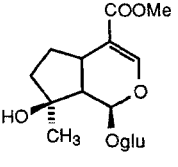
A detailed study of the Michigan Hill population was undertaken to establish firmly what appeared to be a major host-plant utilization difference at the two nearby sites. Such host-plant changes are well-documented for *E. editha* in California (Ehrlich et al., 1975; Singer, 1982, 1983). In addition, our first detailed study focused mainly on variation in the field-captured adult butterfly iridoid content, with less attention paid to host-plant or larval iridoid analyses. Therefore, we also used the Michigan Hill organisms to obtain data on the iridoid content of individual larval-consumed plants and iridoid content comparisons among these plants, larvae, pupae, excretion products and adult butterflies.

METHODS AND MATERIALS

Organisms and Site. Identification of the butterfly, *E. anicia* Doubleday and Hewitson, and the host-plants, *C. integra* Gray and *B. plantaginea* (James) Rydb., was as described previously (Stermitz et al., 1986). The study was con-







1: $R_1 = R_2 = H$ (catalpol)

3: aucubin

7: mussaenoside

2: $R_1 = OH$; $R_2 = H$ (macfadienoside)

4: $R_1 = H$; R_2 = benzoyl (veronicoside)

5: $R_1 = H$; R_2 = 3-hydroxy-4-methoxybenzoyl (isovanillylcatalpol)

6: $R_1 = H$; R_2 = 3,4-dihydroxybenzoyl (verproside)

FIG. 1. Structures of iridoid glycosides.

ducted on a south slope of Michigan Hill, 1 mi NW of US Highway 285 and 2 mi SW of Jefferson, Park County, Colorado (39°22′N, 105°50′W) at 9700 ft (2960 m) altitude. The butterfly population nectars on scattered *Potentilla* (Rosaceae) bushes within an approximately 50-m-wide dry depression and on several species of yellow composites growing alongside a dirt road that bisects the depression. *B. plantaginea* grows in this area with a few *C. integra* and with many more *C. integra* 50–100 m west across a dirt road. East and southeast a broad hill rises approximately 200 m from the ditch. This area of mainly low grasses contains some of the composite nectar plants as well as numerous *C. integra*, but no *B. plantaginea*. The host-plant species generally emerge from early to mid-May and larvae of *E. anicia* (which have overwintered in the second or third instar) can be found on *B. plantaginea* in late May or early June. Adult butterflies generally are flying from middle or late June to late July.

Adult butterflies were collected on July 4 and 26, 1986, in the depression area of the site, immediately placed in an ice chest, and frozen within one to two days. They were held in the freezer and then dried to constant weight for 15–20 hr at 50°C just before analysis. Previous work had shown no difference in iridoid content among fresh, air-dried, or 50° dried butterflies. On May 30, 1987, eight late-instar larvae were collected from plants of *B. plantaginea*, along with leaves from the same plants. The plant material and larvae were analyzed immediately. Twelve additional late-instar larvae were collected on June 7, 1987, from *B. plantaginea* and raised in the laboratory on the host-plant kept fresh in the refrigerator. Seven pupated between two and 15 days after capture. Three died for unknown reasons before pupation, while two did not survive due to parasitization by hymenoptera.

Frass, the meconium emitted after eclosion, and adults were analyzed for iridoid content. Additional *B. plantaginea* were collected on June 7, and these plants were also analyzed for iridoid content. They were used in an experiment designed to compare iridoid content determination on dried vs. fresh *B. plantaginea.*

Analytical Procedures. Iridoid glycoside analyses of adult butterflies, emitted meconium, and pupal cases were performed as previously described in detail (Gardner and Stermitz, 1988). The method essentially consisted of extraction into a polar solvent, clean-up with nonpolar solvent washes, evaporation of the purified mixture to dryness, conversion of the iridoid glycosides in the residue to trimethylsilyl ethers, and final quantitative analysis by gas–liquid chromatography using an internal standard.

Larvae from the field were not given food for 24 hr. They were then weighed, crushed into methanol, and refrigerated until analysis. The MeOH mixture was filtered through tared small polypropylene filters with a small cot-

ton plug into tared Erlenmeyer flasks. After filtration, the filters were dried overnight at 50°C. The MeOH was evaporated in vacuo from the flasks. The combined weight of the material on the filter and the MeOH residue was taken as the dry weight of the larvae. Iridoids in the MeOH residue were quantified by GLC as described previously (Gardner and Stermitz, 1988). Larvae raised to adulthood in the laboratory were individually fed fresh *B. plantaginea* each day. Frass was collected once a week and allowed to dry for later analysis. Pupae were placed in clean small vials with filter paper on the bottom. Six adults (five females and one male) emerged and were frozen 24 hr after emergence. The filter paper containing emitted meconium was shredded and allowed to stand in MeOH for 24 hr before iridoid analysis.

For leaf iridoid analysis, a weighed amount (about 0.30–1.00 g fresh or 0.060–0.30 g dry) was crushed in 20 ml of MeOH and the mixture allowed to stand at room temperature for 24 hr. The mixture was filtered as above through tared filters and into tared flasks. The filters were dried and the MeOH evaporated as above. To the residue 4.00 or 10.00 ml MeOH was added and the mixture was stirred until all residue had redissolved. A 1.00- or 2.00-ml aliquot was removed from the solution and the MeOH evaporated. Choice of quantities depended on the amount of original plant material extracted. Final analysis for iridoids was as previously described.

Two fresh undamaged leaves were obtained from two different plants, and each was cut approximately in half lengthwise. After being weighed, one half of a leaf was extracted immediately with methanol, the methanol was evaporated, and the residue was weighed. The marc remaining after the extraction was allowed to dry and weighed. The sum of the weights of residue and marc established the "dry weight" of the leaf half. Percent volatiles was the difference between fresh and dry weights of the leaf half. The other half of each leaf was weighed, allowed to dry for 14 days at 25°C and reweighed. The difference was the percent volatiles and the remainder the dry weight determined by this method.

RESULTS

Field-Collected Butterfly Analyses. The total iridoid content of the butterflies ranged from 0.5 to 4.3% on a dry weight basis (Tables 1 and 2). Catalpol, **1**, was found in all adults and was the major iridoid in 28 of the 31 analyzed; macfadienoside, **2**, was found in eight of the 31 and was the major iridoid in three of them. Small amounts of aucubin were present in 14 of the butterflies, but always at a very low level (less than 10% of the total iridoid content). Dry weight of the females (28 mg mean) was significantly higher ($P < 0.0001$; Student's t test) than that of males (19 mg mean). The percent total iridoid

TABLE 1. IRIDOID CONTENT OF FIELD-CAPTURED, ADULT *E. anicia*, 1986

	Females					Males				
		Iridoid			Total		Iridoid			Total
Date	Dry wt (mg)	**1** (mg)	**2** (mg)	**3** (mg)	iridoids %	Date	Dry wt (mg)	**1** (mg)	**2** (mg)	**3** (mg)	iridoids %
7/4						7/4					
	19.8	0.19	0.29	0.00	2.5		17.4	0.63	0.10	0.02	4.3
	27.0	0.14	0.24	0.00	1.4		15.0	0.44	0.05	0.00	3.3
	27.1	0.38	0.00	0.00	1.4		23.7	0.13	0.30	0.00	1.7
	28.2	0.28	0.00	0.00	1.0		20.0	0.44	0.00	0.03	2.3
	26.6	0.40	0.00	0.00	1.5		17.9	0.54	0.00	0.00	3.0
	37.2	0.97	0.00	0.00	2.6		23.4	0.70	0.17	0.00	3.7
	35.9	0.26	0.00	0.00	0.7		19.2	0.69	0.00	0.00	3.6
	27.6	0.20	0.00	0.00	0.7						
	31.2	0.85	0.00	0.03	2.8						
	34.0	0.40	0.00	0.00	1.2						
7/26						7/26					
	24.2	0.10	0.00	0.01	0.5		13.4	0.28	0.00	0.00	2.1
	18.3	0.16	0.00	0.00	0.9		15.2	0.39	0.00	0.03	2.8
	29.9	0.88	0.00	0.06	3.1		19.4	0.33	0.00	0.00	1.7
	32.6	0.81	0.00	0.05	2.7		16.7	0.54	0.00	0.04	3.4
	30.1	0.50	0.09	0.02	2.1		16.7	0.21	0.09	0.02	1.9
	20.5	0.47	0.00	0.04	2.5		24.6	0.58	0.00	0.04	2.4
	31.7	0.58	0.00	0.06	2.0						
	26.6	0.61	0.00	0.06	2.5						

TABLE 2. SUMMARY OF *E. anicia* IRIDOID ANALYSES, 1986

		Females								
Date	*N*	Dry wt (mg)	**1** (mg)	**1** (%)	Iridoids (%)	Date	*N*	Dry wt (mg)	**1** (mg)	**1** (%)	Iridoids (%)
7/4	10					7/4	7				
Mean		29.5	0.41	1.3	1.6	Mean		19.5	0.51	2.7	3.1
Median		27.9	0.33	1.1	1.4	Median		19.2	0.54	3.0	3.3
SD[a]		5.2	0.28	0.8	0.8	SD		3.2	0.20	1.1	0.9
7/26	8					7/26	6				
Mean		26.7	0.51	1.9	2.0	Mean		17.7	0.39	2.2	2.4
Median		28.2	0.54	2.0	2.3	Median		16.7	0.36	2.2	2.2
SD		5.3	0.28	0.8	0.9	SD		3.9	0.14	0.7	0.6

[a] Standard deviation.

content of the males was about twice that of the females for the July 4 collection ($P < 0.01$; Student's t-test), but not significantly different for the July 26 collection.

Iridoid Content of *Besseya plantaginea* and Insect Life Stages. Quantitative iridoid analyses performed on the residue from the methanol extraction of the fresh leaves and on a similar extract of the air-dried leaves showed that the percent volatiles and iridoid percentages determined by the two methods were similar, if not identical within experimental error (Table 3). The iridoid content of fresh leaves of six *B. plantaginea* plants varied from 9.1 to 22.3% of the dry weight, while iridoid content of larvae found consuming these plants varied from 7.1 to 18.0% (Table 4). Although the mean content was similar for plants and larvae, there was no direct correlation between the content of individual larvae and their consumed plants. For example, plant 2 had an abnormally high iridoid content and a high catalpol–catalpol ester ratio, but the larva consuming this plant had the lowest iridoid content. Two additional larvae (not in Table 4) were analyzed with similar results: 8.8 and 10.9% total iridoids (catapol and a trace of aucubin, **3**). Plant material for these individuals was not analyzed. One of the eight larvae analyzed contained macfadienoside even though it was collected on *B. plantaginea*. No catalpol esters, major components of the host-plant, were found in the larvae.

Iridoid analyses of frass, pupal cases, meconium, and adult butterfiles from the six field-collected and then lab-raised late-instar larvae showed little total iridoids in the frass and pupal case and relatively large amounts in the meconium and adults (Table 5). Total iridoid content (meconium and adult) was lower than that of the host-plants or larvae (Table 4), but comparable to that of field-collected adults (Table 1). Two of the larvae fed long enough so that frass could be collected in two batches, early and late. Comparison of these batches and

TABLE 3. COMPARISON BETWEEN ANALYSES OF FRESH OR AIR-DRIED *Besseya plantaginea* FOR DETERMINATION OF LEAF VOLATILES AND IRIDOID CONTENT

Leaf	Fresh wt. (mg)	Dry wt. (mg)	Volatiles (%)	Individual iridoid %						Iridoid (%)
				1	**3**	**4**	**5**	**6**	**7**	
1A[a]	362	63	82.6	2.2	0.05	1.1	3.8	3.2	0.1	10.4
1B[b]	319	56	82.4	2.2	0.00	1.1	4.3	3.4	0.1	11.1
2A[a]	292	57	80.5	1.8	0.05	1.7	5.0	4.2	0.1	12.9
2B[b]	345	64	81.6	1.8	0.06	1.3	3.8	3.1	0.1	10.2

[a] One half of leaf extracted fresh.
[b] One half of leaf air-dried, then extracted.

TABLE 4. IRIDOID CONTENT OF SIX *B. plantaginea* PLANTS AND LARVAE COLLECTED FROM EACH PLANT

	Plant and Larva No.						Mean iridoids	
	1	2	3	4	5	6	(%)	SD
Plant iridoids								
Catapol (**1**)	5.1[a]	16.8	3.6	7.2	2.2	9.6	7.4	5.3
Verproside (**6**)	1.8	2.1	3.9	3.1	4.3	3.0	3.0	1.0
Isovanillylcatalpol (**5**)	1.4	1.3	1.5	1.7	0.9	2.0	1.5	0.4
Veronicoside (**4**)	0.7	1.9	1.7	1.1	1.7	1.5	1.4	0.5
Mussaenoside (**7**)	0.4	0.0	0.3	0.0	0.0	0.5	0.2	0.2
Aucubin (**3**)	0.0	0.2	0.1	0.1	0.0	0.2	0.1	0.1
Total iridoid %								
(Plants)	9.4	22.3	11.1	13.2	9.1	16.8	13.7	5.1
(Larvae)[b]	13.6	6.5	7.1	9.5	14.5	18.0	11.5	

[a] Percent, dry weight basis.
[b] All larvae had 0.1 or 0.2% aucubin. Larva 1 had 1.3% macfadienoside and 12.1% catalpol. None had verproside, isovanillylcatalpol, veronicoside, or mussaenoside.

the frass from larvae that pupated within the first days of collection showed that frass from less mature larvae contained some iridoids, indicating a less efficient sequestration than by the more mature larvae. The percent iridoid content of pupal cases was similar to that of the adults, but the dry weight of the cases is so small, they contribute little to the total iridoid content of the organism. One larva, although collected on and fed *B. plantaginea*, showed small amounts of macfadienoside in the frass and meconium.

DISCUSSION

Plant Analyses. Previous iridoid analyses of *B. plantaginea* (Stermitz et al., 1986; Gardner and Stermitz 1988), based upon actual isolation from whole plant material, had indicated a 2–4% iridoid content, although a few individual plant analyses via the GC method showed a much higher content (Gardner, unpublished results). The present work clearly establishes a much higher content (14% mean) when direct analyses by the GC method were conducted on leaf material at the time of larval consumption (Table 4). The iridoids of *B. plantaginea* are apparently stable during air drying of the plant material (Table 3), and hence one need not resort to freezing or immediate solvent extraction when collecting field samples. Individual plants may contain variable amounts of total iridoids, but the general pattern of individual iridoids is relatively con-

TABLE 5. IRIDOID CONTENT OF LAB-RAISED *Euphydryas anicia*

Butterfly number (sex)	Frass		Pupal case		Meconium catalpol (mg)	Adult		Total iridoid (%)
	Weight (mg)	Catalpol (mg)	Weight (mg)	Catalpol[a] (mg)		Weight (mg)	Catalpol (mg)	
1(F)			3.9	0.21	0.05	65	0.87	1.8
3(F)	88	none	2.0	0.08	0.10	53	0.48	1.2
5(F)	20 (early)	0.16			0.71[b]	40	0.26	2.4
	140 (late)	0.08						
6(F)	81	none	3.9	0.44	0.57	58	0.32	2.3
9(M)	82	none	1.4	0.05	0.59	31	0.34	3.2
11(F)	69	0.03	3.6	0.23	0.63	38	0.31	3.1

[a] Pupal cases for butterflies 1, 6, and 11 contained large amounts of meconium.
[b] Pupal case and meconium combined.

sistent from plant to plant (Table 4). An exception was that of plant 2, which had a catalpol content far exceeding the mean. Relative proportions of the catalpol esters **4–6** remained similar for the most part and mussaenoside, **7**, and aucubin were always very minor iridoids.

Butterfly Analyses. Field-collected adults (Table 1) showed fewer (8 of 31) containing macfadienoside than at Red Hill, where 92% in 1985 and 100% in 1986 contained that iridoid (Gardner and Stermitz, 1988). Thus, the major host-plant at Michigan Hill is *B. plantaginea* rather than *C. integra*. While Red Hill is a flat site, with both host-plants occurring together in most quadrants, Michigan Hill contains a variety of terrains and a much patchier distribution of the two hosts. A host-plant count of the Michigan Hill site and butterfly collections at varying portions of the site, such as the hilltopping area, will be needed as a first step to determining whether or not simple host-plant distribution and numbers can account for the different hostplant usage pattern at this site.

Larvae contain large amounts of iridoids (Table 4) relative to the adults, both field collected (Table 2) and lab raised (Table 5). The small total numbers of plants and larvae analyzed (Table 4) preclude a statistical treatment, such as was done recently (Martin and Lynch, 1988) for monarch butterflies and their host plants, but there is no indication of a relationship between larval and host-plant iridoid content. In the monarch case it was suggested that the butterflies were feeding on high cardenolide plants (comparable to the amount they stored) and had reached a saturation level. In our case, the iridoid content of both plant and insect is remarkably high. Catalpol esters represent a major part of the host-plant iridoids, but they were not found in larvae analyzed after 24 hr without food. Thus the esters are rapidly hydrolyzed. There is apparently little or no undigested plant material remaining in the larvae after 24 hr since esters were not found in the larval analyses.

One reason for the high larval content is that little is excreted in the frass (Table 5). There is some indication that very early instar larvae may not be as efficient in sequestration as later ones. Early frass from butterfly 5 showed more catalpol than later frass, although the total amount of frass collected from early instars was small. The meconium excreted upon eclosion contains considerable catalpol, often more than is finally present in the adult (Table 5). Meconium is sometimes left in the pupal case, but where the pupal case is clean iridoids are present even though the contribution to total iridoid content is minimal due to the small pupa case weight. Striking is our failure to correlate the large iridoid content of the larvae with that subsequently found in the adult and meconium, where most should appear. Two last skin casts were checked, but weights were very small and only traces of catalpol were present. This lack of material balance is not restricted to *E. anicia* on *B. plantaginea*, and occurs with some (but not all) other iridoid specialist–host-plant pairs being studied in our laboratories. Both larvae and host plants showed iridoid content comparably high to that

found in the present work, while adults again contain the same substantial, but considerably lower, iridoid content. The best hypothesis may be that metabolism is occurring during the pupal stage in these cases and that such metabolism is converting iridoid glycosides to noniridoid-like materials. Simple conversions such as hydroxylations or similar functional group changes would lead to materials that would show up in our TLC and GLC analyses, but do not. Cleavage of the glucose moiety, however, would lead to an aglycone whose physical, TLC, and GC properties would most likely be sufficiently changed so that it would not be detected in methods developed for glycoside analysis. In addition, iridoid aglycones are much less stable than the glycoside and might further degrade before or during analysis. In contrast to these results, studies on *Poladryas arachne* hosted by *Penstemon virgatus* have shown quite a good iridoid content balance between the consumed plant and life stage totals for the butterfly (L'Empereur, 1989; L'Empereur and Stermitz, in press).

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SAPINDACEAE, CYANOLIPIDS, AND BUGS

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Abstract—Scentless plant bugs (Heteroptera: Rhopalidae) are so named because adults of the Serinethinae have vestigial metathoracic scent glands. Serinethines are seed predators of Sapindales, especially Sapindaceae that produce toxic cyanolipids. In two serinethine species whose ranges extend into the southern United States, *Jadera haematoloma* and *J. sanguinolenta*, sequestration of host cyanolipids as glucosides renders these gregarious, aposematic insects unpalatable to a variety of predators. The blood glucoside profile and cyanogenesis of *Jadera* varies depending on the cyanolipid chemistry of hosts, and adults and larvae fed golden rain tree seeds (*Koelreuteria paniculata*) excrete the volatile lactone, 4-methyl-2(5H)-furanone, to which they are attracted. *Jadera* fed balloon vine seeds (*Cardiospermum* spp.) do not excrete the attractive lactone. Loss of the usual heteropteran defensive glands in serinethines may have coevolved with host specificity on toxic plants, and the orientation of *Jadera* to a volatile excretory product could be an adaptive response to save time.

Key Words—*Leptocoris, Jadera*, Heteroptera, Rhopalidae, Serinethinae, Sapindaceae, cyanogenesis, glucoside, sequestration, allomone, attractant, predation, pheromone, allelochemic.

[4]Mention of a commercial product does not consititute an endorsement by the USDA.

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INTRODUCTION

Rhopalidae (Heteroptera) are called scentless plant bugs because in adults of the Serinethinae the metathoracic scent gland is vestigial. However, this common name is misleading as serinethine adults retain the abdominal scent glands usually found only in immatures, and adults of the Rhopalinae actually do possess a metathoracic scent gland (Aldrich et al., 1979; Aldrich, 1988). Serinethinae prefer Sapindales, especially the Sapindaceae (Schaefer and Chopra, 1982; Schaefer and Mitchell, 1983), where they form aggregations of up to thousands of individuals (Carroll and Loye, 1987; Carroll, 1988; Wolda and Tanaka, 1987). Sapindaceae produce toxic cyanolipids in their seeds (Seigler and Kawahara, 1976); thus loss of the usual heteropteran defensive gland in serinethines may have coevolved with host specificity on these plants. This hypothesis is bolstered by the discovery that the aposematic bug, *Leptocoris isolata*, sequesters host cyanolipids as glucosides and can exude blood containing enough of these compounds to repel ants (Braekman et al., 1982).

Two serinethine species in the genus *Jadera* are common in the southern United States, *J. haematoloma* and *J. sanguinolenta*. In southern Florida, the balloon vine, *Cardiospermum corindum*, is the native host of *Jadera* (Carroll, 1988). Seeds of this plant contain cyanolipid **1** (Scheme 1) (Seigler, 1974; Seigler and Kawahara, 1976). Soapberry, *Sapindus saponaria drummondii*, the native host of *J. haematoloma* in the southwestern United States (Carroll and Loye, 1987), contains only **2** in its seeds (Seigler, 1974). The golden rain tree, *Koelreuteria paniculata*, is an introduced host of *J. haematoloma* in the southwestern and central states, and *K. elegans formosana* has likewise been introduced in Florida, where its seeds are fed upon by both *J. haematoloma* and *J. sanguinolenta* (Carroll and Loye, 1987; Carroll, 1988). *K. paniculata* seeds contain **2** and **3** (Mikolajczak et al., 1970) and *K. e. formosana* probably produces a similar cyanolipid blend (Seigler and Kawahara, 1976). We tested the acceptability of *Jadera* as prey for various predators and examined the sequestration of glycosides of cyanolipids in *Jadera* as a function of host species. Our results provide an insight into the chemical vocabulary of these semisocial bugs.

METHODS AND MATERIALS

Insects. Jadera spp. were collected by S.P.C. in 1985, and express-mailed to J.R.A. for dissection or rearing. Cultures of *J. haematoloma* and *J. sanguinolenta* were maintained on *K. paniculata* seeds for several generations and for one generation on *C. corindum* and *C. grandiflorum* seeds, plus water.

Two lycaenids (Lepidoptera) feeding on unripe seeds of *C. corindum* were

collected by S.P.C. at Plantation Key, Florida, in February 1988 and sent to J.R.A. for analysis: *Hemiargus thomasi* and *Clorostrymon simaethis*.

Chemical Analysis. Insect blood was collected in capillary tubes from amputated legs. Methanolic extracts were acetylated in pyridine–acetic anhydride (1 : 1), fractionated on silica gel, and monitored by TLC (silica gel 60 F_{254} nano plates). Gas chromatography (GC) was performed using a Varian 3700 GC with a Shimadzu C-R3A recorder, on a 15-m bonded methyl silicone capillary column (0.25 mm ID; DB-1, J&W Scientific, Folsom, California) with helium as carrier (40 cm/sec) at 145°C for 2 min to 240°C at 15°/min. Urine from bugs was extracted with methylene chloride (20 μl/100 μl CH_2Cl_2), and analyzed by GC (DB-1, 45°C for 2 min to 230°C at 15°/min). The volatile excretory product and acetylated glycosides were isolated in glass capillary tubes from a DB-1 column (15 m × 0.53 mm ID) in a Varian 3700 GC equipped with a thermal conductivity detector. GC–mass spectrometry was conducted using a Finnigan 4510 GC-MS system on a 30-m DB-1 column. NMR spectra were recorded at 60 MHz on a JEOL FX-60Q FT instrument and at 300 MHz on a General Electric QE-300 instrument with TMS as an internal standard. Decoupling experiments were performed for some samples at 300 MHz using single-frequency, low-pressure, on-resonance conditions to remove the target absorption. Infrared spectra were obtained in CCl_4 using a Perkin-Elmer 580B spectrometer and a UV spectrum of **4** (Figure 2 below) was recorded on a Perkin-Elmer 559 UV-VIS spectrometer. 4-Methyl-2(5H)-furanone (**4**) was synthesized according to published procedures (Price and Judge, 1973; Pelletier et al., 1975).

Cyanogenesis. A cupric acetate–benzidine acetate test solution on filter paper strips was used to check for release of HCN from crushed bugs and urine (Feigl, 1966). β-Glucosidase (40 μl, 4 mU/μl, 0.1 mM citrate/PO_4 buffer, pH 5.00; Sigma) was also added to preparations in conjunction HCN indicator tests.

Attraction Bioassay. Insects were anesthetized with CO_2 and placed in a 500-ml three-neck flask. A 250-ml splash-guard adapter was attached to each side arm, and the side arms were connected via silicone tubing to charcoal filtered and humidified air. The middle neck of the flask was connected to the house vacuum (20 ml/min), and the apparatus was positioned horizontally on a countertop in a room (23 ± 2.5°C) with bright fluorescent lights. Five, 10, or 50 μl of **4** (10 μg/μl in CH_2Cl_2) was applied to filter paper in the upwind end of one splash-guard and the other arm had filter paper with CH_2Cl_2 only. Treated and control sides were alternated between tests. The number of insects in each splash-guard was counted every 15 min for the first 2 hr, then hourly, and the next morning.

Predator Aversion. Jadera haematoloma from golden rain trees were offered to four toad species (*Bufo woodhousei, B. americanus, B. cognatus,*

and *B. speciosus*), the blue jay (*Cyanocitta cristata*), and praying mantids (probably Chinese mantids, *Tenodera ardifolia sinensis*).

RESULTS

Identification of Volatile Excretory Product. Urine from *J. sanguinolenta* adults or larvae fed *K. paniculata* seeds had a single volatile component (Figure 1A, **4**); bugs fed balloon vine seeds had no volatiles (Figure 1C). *J. haematoloma* adults and larvae also excreted **4** when fed golden rain tree seeds, but not when fed balloon vine seeds. The MS of **4** suggested a methyl-2(5H)-furanone structure. EI-MS *m/z* (%): 98(M^+, 33), 70(5), 69(100), 68(11), 55(1), 55(3), and 50(3); CH_4 CI-MS: 99($[M+H]^+$, 100); NH_3 CI-MS: 214($[2+NH_4]^+$, 2), 150($[M+(NH_3)_3H]^+$, 10). 133($[M+(NH_3)_2H]^+$, 100), and 116($[M+NH_4]^+$, 35). The identity of **4** as 4-methyl-2(5H)-furanone (3-methyl-2-butenolide) was deduced from spectral data: UV γ_{max} nm: 210; IR (CCl_4): γC=O 1755/1785 cm^{-1}, γC=C 1650 cm^{-1}; [^{1}H]NMR (60 MHz, $CDCl_3$) δ 2.16 (br s, 3H), 4.75 (br s, 2H), and 5.89 (br s, 1H); lit. (Liardon and Philippossian, 1978): IR (film): γC=O 1740/1775 cm^{-1}, δC=C 1640 cm^{-1}; [^{1}H]NMR (60 MHz, $CDCl_3$) δ 2.18 (m), 4.81 (m), and 5.94 (m). Insect-derived **4** coeluted with synthetic **4** by GC.

Identification of Blood Glycosides. Pinching the bugs usually caused them to bleed intersegmentally and eject scent secretion. Bleeding was most prevalent at the rostrum and, in adults, from the openings of the metathoracic scent gland. Methanol extracts of exuded fluid and of hemolymph showed the same components by TLC ($CHCl_3$–ethanol, 60:40) indicating that the externalized fluid is blood. Acetylation of 20 μl of blood extract from *J. sanguinolenta* larvae fed *K. paniculata* seeds confirmed by GC (Figure 1B) the pattern observed by TLC; compounds **6**, **8**, **12**, and **14** accounted for 0.5, 2.5, 88, and 4.5% of the total peak area, respectively. GC of an acetylated extract from *J. sanguinolenta* larvae reared on balloon vine seeds exhibited a new component (**10**, 69%) in addition to **12** (26%), and compounds **6**, **8**, and **14** were absent (Figure 1D).

The major blood component from six larvae and six adults of *J. haematoloma* reared on *K. paniculata* seeds (silica gel; 3 ml fractions of 10, 20, 40, 60, and 80% methanol–$CHCl_3$) gave CI-MS consistent with a monoglycoside having an aglycon of mol wt 275. The presence of the nitrile group in **11** was confirmed by the IR spectrum (γC≡N at 2228 cm^{-1}) and, since cardiospermin (=**9**) does not show IR absorption in the 2250 cm^{-1} region (Seigler et al., 1970, 1974), this structure was ruled out. The identity of **11** was confirmed by comparison to acetylated 4-β-D-glucopyranosyloxy-3-hydroxymethyl-2-butenylnitrile (=**12**, Figure 2) prepared from material isolated from *L. isolata*.

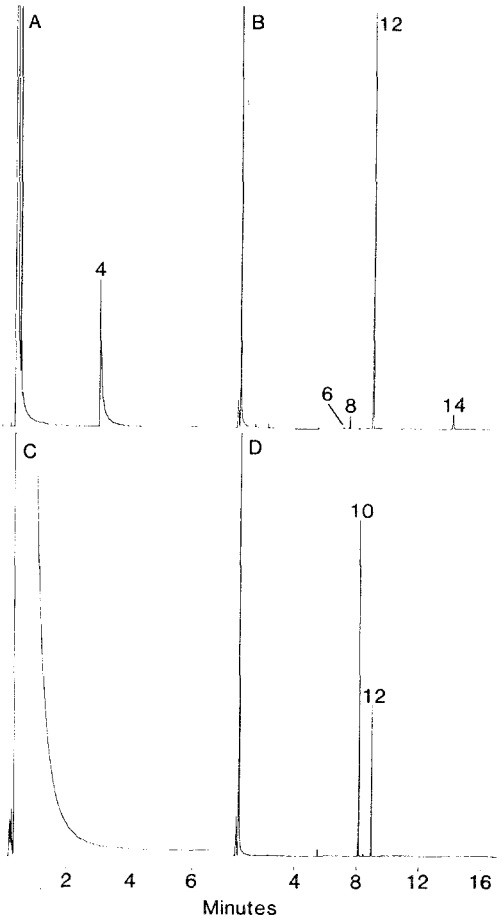


FIG. 1. Gas chromatograms of CH_2Cl_2 extracts of excreta and acetylated MeOH blood extracts from *Jadera sanguinolenta* adults reared on hosts with different cyanolipids (numbering of peaks follows Figure 2): (A) fed *Koelreuteria paniculata*/urine; (B) fed *K. paniculata*/blood; (C) fed *Cardiospermum cornindum*/urine (splitless GC injection); (D) fed *C. corindum*/blood.

For identification of minor bloodborne glycosides of *Jadera* reared on *K. paniculata* seeds, blood was collected from 166 male and female *J. sanguinolenta* in 3 ml of methanol and acetylated. Fractions (5 g silica gel) with material migrating above **11** by TLC were combined, as were those with material migrating below **11**. There were two prominent compounds in the fast-eluting fractions, one with an NH_3 CI-MS base peak at *m/z* 447 corresponding to a mol wt

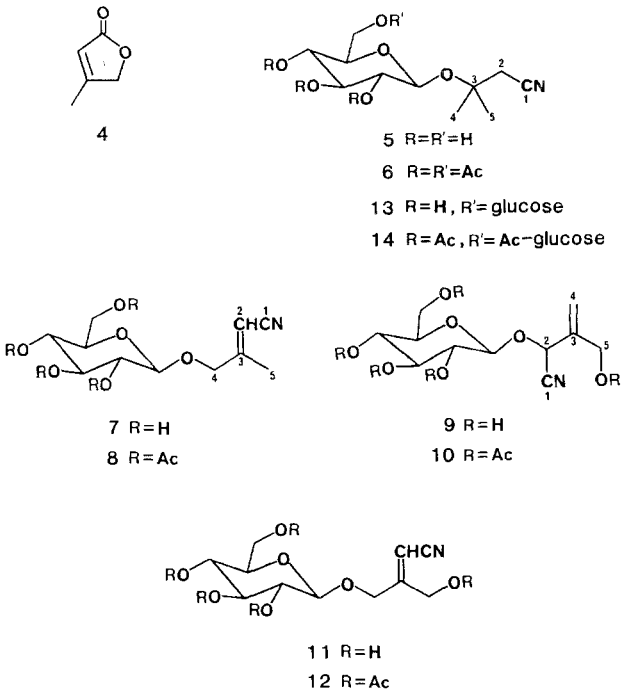


FIG. 2. Strucures of *Jadera* excretory lactone and blood-sequestered glucosides.

429 (Figure 1B, **6**) and the other with a base peak at *m/z* 445 corresponding to a mol wt 427 (Figure 1B, **8**). The [^{1}H]NMR of **8** differed characteristically from that of **12** by the absence of the H_2C-5 signal and the upfield shift of one methyl signal. Structure **7** (Figure 2) is proposed for this minor glycoside apparently derived from cyanolipid **3**. Compound **14** was isolated from the slow-eluting material by refractionating on silica gel (1:1, 3:1, and 6:1 $CHCl_3$–benzene) and recrystallizing from acetone–hexane (6:1 $CHCl_3$–benzene fraction). The NH_3 CI-MS of **14** exhibited a base peak at *m/z* 735 indicating a mol wt 717. This was substantiated by the CH_4 CI-MS (*m/z* (%) 718(20), $[M+H]^+$), and ions at *m/z* (%) 619(35) and 331(100) suggested the presence of a dihexose moiety. Two anomeric proton doublets (δ 4.57, J = 7.8 Hz; δ 4.70, J = 8.0 Hz) were observed in the [^{1}H]NMR, indicating a β-linked diglycoside structure. The signal at δ 4.08–4.30 integrated to two protons (H_2C-6″) whereas the signal at δ 3.58–3.84 integrated to four protons (HC-5′, HC-5″, and H_2C-6′), thus a β(1–6)disaccharide linkage was indicated. This interpretation was substantiated by comparisons to the [^{1}H]NMR spectra of amygdalin, sophorose, and melibiose. The EI-MS of **14** exhibited ions at *m/z* 702 ($[M-CH_3]^+$, <1%) and

677([M-CH_2CN]$^+$, 1%) consistent with a 3-glycoside of 3-methylbutyronitrile (Spencer and Daxenbichler, 1980). Decoupling experiments ruled out an aglycon corresponding to saturated **7** in favor of the dimethyl aglycon. Based on the CI-MS of **6** and the similarity of the EI-MS of **6** to that of **14**, structure **5** is proposed as the monoglucoside analog of **13**.

Compound **10** was the major glycoside of an acetylated methanolic extract of blood from *J. sanguinolenta* larvae reared on seeds of *C. corindum* (Figure 1D). The NH_3 CI-MS of **10** exhibited a base peak at *m/z* 503 indicating a mol wt 485, and the EI-MS showed an *m/z* 331 (9%) ion indicative of a hexose moiety. The IR of the isolated compound had no $\gamma C{\equiv}N$ and the [^{1}H]NMR spectrum had a pair of singlets at 5.54 δ and 5.66 δ (vinyl, 2H), a singlet at 5.20 δ (cyanohydrin H), and was otherwise identical to the published spectrum of acetylated cardiospermin (Seigler et al., 1974).

Aliquots of blood (5–15 μl) from the two lycaenid caterpillars collected from *C. corindum* showed no trace of the glycosides characteristic of *Jadera* feeding on balloon vine seeds.

3-Glucopyranosyloxy-3-methylbutyronitrile (5). Characterized as the tetraacetate **6**. NH_3 CI-MS *m/z* (%): 447 ([M+NH_4]$^+$, 100); $^{15}NH_3$ CI-MS: 448([M+$^{15}NH_4$]$^+$, 100); EI-MS: 331([M-C_5H_8ON]$^+$, 4), 242(26), 200(30), 169(33), 157(73), 140(39), 115(100), 98(90), 82(51), and 55(28).

4-β-Glucopyranosyloxy-3-methyl-2-butenylnitrile (7). Characterized as the tetraacetate **8**. NH_3 CI-MS *m/z* (%): 445([M+NH_4]$^+$, 100); $^{15}NH_3$ CI-MS: 446([M+$^{15}NH_4$]$^+$, 100); EI-MS: 331([M-C_5H_6ON]$^+$, 3), 265(9), 243(15), 200(23), 169(33), 157(49), 145(50), 112(65), 98(100), 81(97), 69(45), and 53(59); [^{1}H]NMR (300 MHz, $CDCl_3$) δ 1.95 (s, H_3C-5), 2.01–2.10 (4s, 12H), 3.72 (m, HC-3′), 4.16 (dd, HC-6′), 4.27 (dd, HC-6′), 4.48 (br s, H_2C-4), 4.52 (d, J = 7.8, HC-1′), 5.04 (dd, HC-2′), 5.10 (dd, HC-4′), 5.22 (t, HC-3′), and 5.27 (br s, HC-2).

Cardiospermin (2-β-glucopyranosyloxy-3-hydroxymethyl-3-butenylnitrile) (9). Characterized as the pentaacetate **10**. NH_3 CI-MS *m/z* (%): 503([M+NH_4]$^+$, 100); EI-MS: 331([M-$C_7H_8O_3N$]$^+$, 9), 271(2), 229(2), 211(3), 169(100), 138(19), 127(27), 109(92), 97(17), 81(15), and 69(11); IR (CCl_4) no $\gamma C{\equiv}N$; [^{1}H] NMR (300 MHz, $CDCl_3$) δ 2.02–2.10 (5s, 15H), 3.78 (m, HC-5′), 4.18 (dd, HC-6′), 4.27 (dd, HC-6′), 4.65 (br s, H_2C-5), 4.83 (d, J = 7.8, HC-1′), 5.03–5.14 (m, HC-2′, HC-4′), 5.20 (br s, HC-2), 5.26 (t, HC-3′), 5.34 (br s, HC-4), and 5.66 (br s, HC-4).

4-β-Glucopyranosyloxy-3-hydroxymethyl-2-butenylnitrile (11). NH_3 CI-MS (probe) *m/z* (%): 310([M+$(NH_3)_2$H]$^+$, 42), 293([M+NH_4]$^+$, 100), 215([hexose+$(NH_3)_2$H]$^+$, 26), and 198([hexose+NH_4]$^+$, 15); $^{15}NH_3$ CI-MS: 312(92), 294(100), 217(78), and 199(86); ND_3 CI-MS: 322(39), 302(100), 227(55), and 207(55); CH_4 CI-MS: 276([M+H]$^+$, 2); isobutane CI-MS: 332([M+C_4H_9]$^+$, 57), and 276(100); EI-MS: 155(2), 114(8), 97(16), 85(16),

73(72), 67(68), 60(100), and 57(51); IR(CCl_4): $\gamma C{\equiv}N$ at 2228 cm^{-1}. Further characterized as pentaacetate **12**. NH_3 CI-MS *m/z* (%): 503($[M+NH_4]^+$, 100); EI-MS: 331(2), 323(4), 292(2), 250(4), 242(11), 200(17), 169(25), 157(40), 145(37), 138(100), 115(45), 112(54), 98(58), 81(68), 70(34), 69(35), 61(7), and 52(6); [^{1}H]NMR (300 MHz, $CDCl_3$) δ 2.01–2.14 (5s, 15H), 3.73 (m, HC-5′), 4.17 (dd, HC-6′), 4.26 (dd, HC-6′), 4.55(d, J = 8.1, HC-1′), 4.59 (br s, H_2C-4), 4.74 (t, H_2C-5), 5.02 (dd, HC-2′), 5.09 (dd, HC-4′), 5.21 (t, HC-3′), and 5.54 (br s, HC-2); [^{13}C]NMR (300 MHz, $CDCl_3$) δ 20.0–20.2 (5C, $\underline{C}H_3COO$), 61.2 (C-6′), 62.3 (C-4), 66.6 (C-5), 67.7 (C-4′), 70.5 (C-2′), 71.8 (C-3′ or 5′), 72.2 (C-3′ or 5′), 97.2 (C-2), 99.6 (C-1′), 114.3 (C-1), 155.8 (C-3), and 168.8–170.1 (5C, $CH_3\underline{C}OO$). As the shift for the nitrile carbon (C-1) was at variance with that reported by Braekman et al. (1982) (δ 120.7), a [^{13}C]NMR (60 MHz, $CDCl_3$) spectrum of **12** derived from *L. isolata* supplied to us by Dr. Daloze was obtained on our instrument; the nitrile signal occurred at δ114.6. EI- and CI-MS of **12** from *L. isolata* were identical to **12** derived from *Jadera*.

*6-O-β-Glucopyranosyl-3β-glucopyranosyloxy-3-methyl-butyronitrile (**13**).* Characterized as heptaacetate **14** (2.6 mg, mp = 197–197.5°C). NH_3 CI-MS *m/z* (%): 735($[M+NH_4]^+$, 100); ND_3 CI-MS: 739(100); CH_4 CI-MS: 746($[M+C_2H_7]^+$, 9), 718($[M+H]^+$, 17), 619([dihexose]$^+$, 33), and 331([hexose]$^+$, 100); EI-MS: 677($[M\text{-}CH_2CN]^+$, 1) 370(2), 331(59), 317(16), 271(8), 215(11), 169(100), 157(15), 127(20), 109(51), 97(21), 81(48), 69(29), and 55(25); [^{1}H]NMR (300 MHz, $CDCL_3$) δ 1.39 and 1.40 (2s, H_3C-4 and H_3C-5), 2.00–2.10 (7s, 21H), 2.56 (d, J = 2.4, H_2C-2), 3.65 (d, HC-6′), 3.61–3.75 (m, HC-5′, HC-5″), 3.81 (dd, HC-6′), 4.12 (dd, HC-6″), 4.27 (dd, HC-6″), 4.57 (d, J = 7.8, HC-1′), 4.70 (d, J = 8.1, HC-1″), 4.85–5.09 (m, 4H; HC-4′, −4″, −2′, −2″), and 5.16–5.24 (m, HC-3′, HC-3″). [^{1}H]NMR (200 MHz, $CDCL_3$) of 3-methyl-3-(trimethylsiloxy)-butyronitrile lit. (Imi et al., 1987): 0.15 (s, 9H), 1.41 (s, 6H), and 2.47 (s, 2H).

Cyanogenesis. When five larvae and five adults of *J. haematoloma* reared on *K. paniculata* seeds were crushed separately in 4-ml vials, no HCN was detected even after addition of H_2SO_4. Single *J. haematoloma* larvae fed either *K. paniculata* or *C. grandiflorum* seeds were crushed in vials containing indicator paper and, after 10–15 min 40 μl of a β-glucosidase solution was added. Bugs reared on *K. paniculata* seeds showed no indication of HCN, and addition of β-glucosidase did not produce HCN. Larvae fed *C. grandiflorum* seeds tested negatively for HCN at first, but positively after addition of β-glucosidase. Urine (ca. 3 μl) collected from larvae fed *C. grandiflorum* was not cyanogenic even when β-glucosidase was added, nor was urine from larvae fed golden rain tree seeds. Last-instar *J. sanguinolenta* larvae were also tested as just described; *Cardiospermum*-fed bugs tested positively for HCN upon addition of β-glucosidase, but *K. paniculata*-fed bugs were acyanogenic within the observational time period (ca. 30 min).

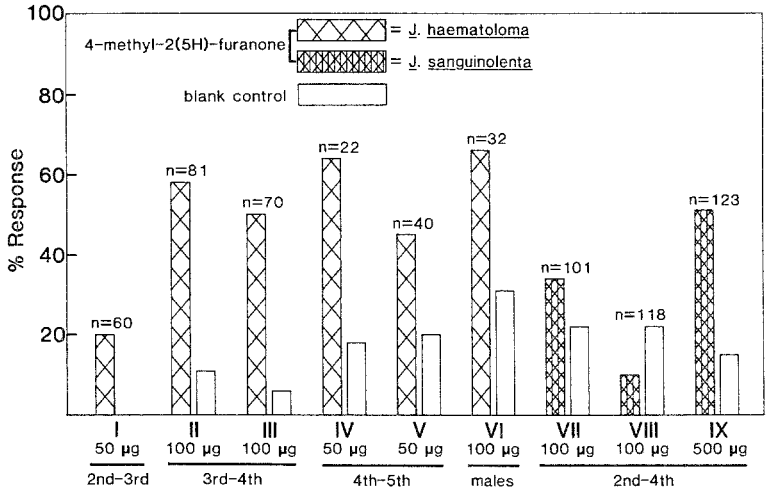
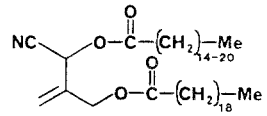
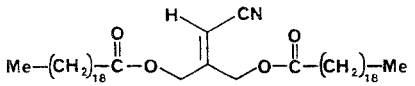


FIG. 3. Attraction of *Jadera* to 4-methyl-2(5H)-furanone.

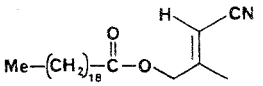
Attraction Bioassay. J. haematoloma were attracted to **4** at the 50- and 100-µg dosages (Figure 3, tests I–VI; $X_1^2 > 3.84$, $p < 0.05$). Maximum response was usually recorded the morning after the test was started. For test I the maximum response occurred 2 hr after starting the test, but by the next morning the numbers of larvae in the treated versus control arms were not significantly different and the overall response was the lowest of all the *J. haematoloma* tests. Responses of *J. sanguinolenta* larvae to **4** were low at the 100-µg dosage and equivocal; in test VII the response was not significant from the control, and in test VIII more larvae occurred in the control arm (Figure 3). At a dose of 500 µg, *J. sanguinolenta* larvae were significantly attracted to **4** (Figure 3, test IX; $X_1^2 = 25.00$).



1



2



3

SCHEME 1.

Predator Aversion. Naive toads ate all 40 larvae offered, but later ate only two of 40 larvae and four of 40 adults (while readily eating mealworms) (Ribiero, 1989). Naive toads that initially ate adult bugs did not later avoid adults: 22 of 32 were eaten. However, toads ingesting adults in the second trial wiped their mouths and eyes and attempted to regurgitate. In tests with five captive, naive blue jays, each bird ate some or all of the three to four late-stage larvae offered initially, but only two ate bugs in a second trial; in a third trial, neither of these two birds would feed on bugs. One bird regurgitated after eating bugs. Experienced birds often tossed the bugs out of their cages. *Jadera* larvae offered to praying mantids were grasped and mandibulated for <5 sec and then thrown from the tree. Three larvae showed signs of pronotal damage, but none were permanently harmed. All five rejected larvae-emitted scent gland secretion and none of the mantids would accept another bug.

DISCUSSION

Serinethines are vividly marked, red insects that suck the seed oil of toxic Sapindales. They lack the stink glands common to most adult Heteroptera (hence the misnomer), yet still secrete, excrete, and bleed an impressive array of semiochemicals. A predator faces irritating vapors of α,β-unsaturated aldehydes, keto-aldehydes, and monoterpenes and, if it persists, is likely to have its mouth smeared with blood containing cyanogenic glucosides. For color-blind predators like shrews (Insectivora) (Huheey, 1984), scent gland odors may serve as aposematic signals associated with bloodborne toxins.

Our results show that HCN is released from crushed *Jadera*, but only in the presence of a β-glucosidase and only if the bugs are reared on balloon vine seeds. Sequestration of the glucoside cardiospermin by *Jadera* contrasts strikingly with the absence of this compound from the blood of lycaenid caterpillars that feed on balloon vine in which the concentration of cardiospermin is high (cf. Braekman *et al.*, 1982). Glucosides accumulated in the blood of *Jadera* fed soapberry or golden rain tree seeds are not truly cyanogenic; however, even acyanogenic cyanolipids exhibit insecticidal activity (Mikolajczak et al., 1984), and many predators reject bugs containing glucosides of these compounds.

The specialized *Jadera* mode of life is further highlighted by the ease with which the bugs exude blood. This capability is also reported for the serinethine *L. isolata* (Daloze et al., 1982) and is well known for *Oncopeltus fasciatus* (Heteroptera: Lygaeidae) and certain other lygaeine seed predators of cardenolide-containing Apocynales (Scudder et al., 1986). Notwithstanding the congeneric status of *L. isolata* and *L. trivittatus* (the boxelder bug), the latter species failed to ooze blood even after very rough handling (Aldrich, unpublished). In

fact, the glycoside chemistry of the former species is much more closely allied to that of *Jadera* than boxelder bugs. Certainly this is because *L. isolata* and *Jadera* feed on sapindaceous plants.

Analysis of the excreta of *J. haematoloma* and *J. sanguinolenta* for volatiles revealed a heretofore unknown dietary effect with intriguing ecological ramifications. 4-Methyl-2(5H)-furanone (**4**) is excreted by adults and larvae only if the bugs feed on golden rain tree (or soapberry?) seeds, and the bugs are attracted to this lactone. In southwestern North America, soapberry and golden rain trees typically mature thousands of seeds per tree at once in August and September, compared to the more continuous maturation of usually no more than several hundred seeds for balloon vine in the Florida Keys (Carroll, 1988). These phenological idiosyncrasies apparently have been paramount for evolution of *J. haematoloma* adults in Florida that are larger and produce larger eggs that take longer to hatch than do *Jadera* from Oklahoma (Carroll, 1988). For populations of Serinethinae on soapberry and golden rain trees, the limiting "resource" may be time rather than food (Carroll, 1988), so the orientation of *Jadera* individuals to a volatile excretory product could be an adaptive response to save time.

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TOXINS IN CHRYSOMELID BEETLES
Possible Evolutionary Sequence from De Novo Synthesis to Derivation from Food-Plant Chemicals

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Abstract—In the Chrysomelinae, it appears that de novo synthesis of chemicals for defense is the primitive state, and the sequestration of plant chemicals for defense the derived state. The derived state evolved through both the morphological and biochemical preadaptiveness of the homologous defensive glands. In the adults, we discuss one unique case of sequestration in exocrine defensive glands of host-plant pyrrolizidine alkaloids by *Oreina cacaliae*. However, hypericin is not sequestered either in the glands or elsewhere in the body of *Chrysolina* spp. feeding on *Hypericum*, which contradicts an earlier claim. In the larvae, we examine in more detail how the phenolglucoside salicin can be used as the precursor of the salicylaldehyde present in the defensive secretion of *Phratora vitellinae* and *Chrysomela* spp. with minimal changes in the biochemical mechanisms involved in the biosynthesis of iridoid monoterpenes in related species.

Key Words—Coleoptera, Chrysomelidae, beetle, sequestration from plants, defense, pyrrolizidine alkaloids, salicin, salicylaldehyde, iridoid monoterpenes, hypericin, *Oreina cacaliae*, *Phratora vitellinae*, *Chrysomela* spp.

INTRODUCTION

Leaf-beetles provide us with striking examples of brilliant coloration. In many chrysomelid species, this conspicuousness is clearly fortified by unpalatability derived from chemical defense in both larval and adult stages. Upon alarm, these defensive chemicals can be released from specialized exocrine glands, by reflex bleedings, or by enteric discharges. In some cases, it is the mechanical action of the predator itself that causes the defensive substance to ooze from wounds of the prey.

The morphology, biology, and chemistry of such defenses in the Chrysomelidae has recently been reviewed (Pasteels et al., 1988) and will not be discussed in further detail here. The major point of interest in this paper is the derivation of chemical defense from host plants from a biochemical as well as evolutionary point of view. The Chrysomelidae are a relatively well-studied group that are known to biosynthesize their own chemical defenses (e.g., iridoids, cardenolides, isoxazolinones) (Pasteels et al., 1988) or to sequester their defenses from a food plant (e.g., salicylaldehyde, juglone, cucurbitacins) (Ferguson and Metcalf, 1985; Pasteels et al., 1989a). This base of comparative knowledge provides a platform from which we can begin to assess the processes involved in the evolution of the diversity of chemical defenses found in the taxon.

Here we review our recent work on plant-derived toxins in the defensive secretion of the Chrysomelinae. More particularly, we postulate the biochemical mechanisms that have allowed the replacement of autogenous compounds by plant-derived toxins by some taxa in the course of evolution.

Some general information on the nature and occurrence of the defensive glands is required before a more detailed discussion of the derivation of defense can be given. Defensive exocrine glands are found in both larval and adult leaf beetles. However, these two sets of glands are not homologous, and their distribution is restricted to certain taxa (Deroe and Pasteels, 1982; Pasteels et al., 1988). Moreover, when they occur in both larval and adult stages of the same species, they secrete toxins of completely different chemical natures. So far, no species is known in which plant-derived toxins occur in the defensive glands of both larvae and adult defensive glands. Yet, in both adults and larvae of genetically closely related species, the same morphological structures are utilized either to synthesize autogenous toxins or to store plant-derived toxins. This genetic relatedness, in conjunction with differences in the identity of chemical defenses, particularly in homologous glands, provides an opportunity to determine the influence of the host plant on defensive chemistry.

In the first part of this paper we report on a previously undescribed and striking example of host-plant influence on the exocrine secretions of adult Chrysomelinae (*Oreina* and pyrrolizidine alkaloids) and reconsider briefly a

previous report of sequestration (*Chrysolina* and hypericin). In the second part, we examine how plant toxins are sequestered in larval secretion (e.g., *Phratora* and *Chrysomela*, and phenolglucosides) and relate this to the probable mode of evolution of this habit.

HOST-PLANT INFLUENCE ON ADULT DEFENSE

In adults of the subfamily Chrysomelinae, the defensive glands are situated in the pronotum and the elytra, especially along the lateral margins. After physical disturbance, the secretion oozes from the gland pores and collects in marginal grooves as well as pronotal and elytral depressions, which help to retain the secretion on the insect. Each gland is formed from a group of secretory cells opening into a common duct. The secretion accumulates within the gland cells themselves, as there is no glandular reservoir. Within this taxon, these glands are morphologically very similar and undoubtedly homologous (Deroe and Pasteels, 1977, 1982). Comparative chemical studies have demonstrated a remarkably diversified defensive chemistry, including cardenolides, dipeptides, and isoxazolinone glucosides. The types of defensive compounds found in the different subtribes studied are summarized in Table 1. There is a close parallel between current classification and the type of compound secreted (see Pasteels et al., 1988).

In this section we will consider only the adults of the subtribe Chrysolinina, which, with few known exceptions to date, produce cardenolides and ethanolamine. In most species of the two European genera of this subtribe, the secretion contains a diversity of cardenolides (Table 2). The structure of 22 different cardenolides has been elucidated (Van Oycke et al., 1988). These cardenolides differ either in their aglycones, of which six are structurally identified,

TABLE 1. DISTRIBUTION OF ADULT DEFENSIVE COMPOUNDS MOST FREQUENTLY FOUND IN SUBTRIBES OF CHRYSOMELINAE (Chrysomelidae, Coleoptera)

Chrysomelinae subtribes	Defensive compounds
Chrysolinina 2 genera, 21 spp.	Cardenolides and ethanolamine
Doryphorina 1 sp.	γ-Glutamyl dipeptide
Chrysomelina 6 genera, 9 spp.	Isoxazolinone and nitropropionic acid glucosides
Phyllodectina 1 genus, 3 spp.	

TABLE 2. DEFENSIVE CHEMISTRY OF ADULT CHRYSOLININA PRODUCING CARDENOLIDES

Beetles	Host plants	Secretions
Oreina	Apiaceae	22 cardenolides identified
8 spp.	Asteraceae	6 aglycones
		8 sugars or esterified derivatives
Chrysolina	Lamiaceae	
13 spp.	Ranunculaceae	up to 138 μg/insect
	Plantaginaceae	(2×10^{-1} M to 4×10^{-1} M)
	Scrophulariacae	also present: ethanolamine
	Hypericaceae	

or in their sugar moieties, of which eight have been elucidated. Cardenolides are found in at least eight species of *Oreina* and 13 species of *Chrysolina* (Pasteels et al., 1988). This widespread occurrence suggests that this is the plesiomorphic condition in the taxon. The host plants of the beetles belonging to these two genera are quite diverse, including seven different families of plants (Table 3). None of these host plants contains cardenolides. Van Oycke et al. (1987) were able to demonstrate that in *C. coerulans* the cardenolides are biosynthesized from cholesterol, following a metabolic pathway that is identical to that found in plants. The function of ethanolamine may be to increase the solubility of the cardenolides in the secretion to reach concentration greater than 10^{-1} M.

TABLE 3. HOST-PLANT RELATIONSHIPS AND CHEMICAL DEFENSES OF ADULT CHRYSOLININA

Beetles	Host plants	Secretions[a]
12 spp. of *Chrysolina*	*Mentha, Lycopus, Rosmarinus, Galeopsis, Glechoma, Ranunculus, Plantago, Linaria*	Cardenolides
Chrysolina dydimata	*Hypericum*	Cardenolides
3 other spp. of *Chrysolina*	*Hypericum*	6-Oxosteroids
7 spp. of *Oreina*	*Chaerophyllum, Peucedanum, Centaurea*	Cardenolides
Oreina speciosissima	*Senecio, Adenostyles, Petasites*	Cardenolides
Oreina cacaliae	*Senecio, Adenostyles, Petasites*	Pyrrolizidine alkaloids

[a] Ethanolamine is also present in all secretions independently of the type of compound secreted.

There are, however, two striking exceptions to this pattern: three species of *Chrysolina* feeding on *Hypericum* secrete 6-oxosteroids and one species of *Oreina* feeding on various Asteraceae secretes pyrrolizidine alkaloids, in addition to ethanolamine. None of these insects secrete cardenolides.

In *Oreina cacaliae*, the major component of the secretion is seneciphylline *N*-oxide (Pasteels et al., 1989b). This species feeds on three genera of Asteraceae, all known to contain the pyrrolizidine alkaloid seneciphylline in its *N*-oxide form. Although sequestration has not yet been demonstrated unambiguously, the presence of exactly the same compound in the plant and in the beetle is unlikely to be a mere coincidence. Thus, secretion of autogenous cardenolides appears to have been replaced by sequestration of plant toxins; but ethanolamine is still produced by the beetle. More mystifying is *O. speciosissima*, a closely related sympatric species, which feeds on the same set of plants but secretes cardenolides rather than pyrrolizidine alkaloids. There are at least three possible alternative and not exclusive hypotheses to account for this. First, it may be advantageous to keep the secretions as chemically diverse as possible to avoid adaptation of specialized predators. Second, the secretion of cardenolides may be the more primitive condition, and thus, *O. speciosissima* has not yet developed the ability to sequester plant toxins. Thirdly, the defensive secretions may also have some species-specific pheromonal function.

Three of four species of *Chrysolina* feeding on *Hypericum* form the second exception to the rule of secretion of autogenous cardenolides (see Table 3). *Chrysolina* feeding on *Hypericum* produce as their main chemical defense polyoxygenated steroids characterized by a ketonic function on carbon 6 as in ecdysone (Daloze et al., 1985; Randoux et al., in preparation). The metabolic origin of these compounds is still unknown, although it is reasonable to hypothesize that they are metabolites of sequestered plant sterols.

There is a persistent claim in the literature that *C. brunsvicensis* sequesters the plant quinone hypericin. This claim is based on a short report by Rees (1969), which states that the quantity of hypericin found in the beetle was too high to be contained solely in the gut. Hypericin, however, is present neither in the defensive secretions containing polyoxygenated steroids, nor is it present in the hemolymph or other body parts of *C. brunsvicensis*, *C. varians*, *C. hyperici*, and *C. geminata* in amounts that could significantly contribute to defense (<0.5 μg/beetle) (Duffey and Pasteels, in preparation).

Thus, it seems that certain beetle species have evolved to replace their primitive de novo secretion with sequestered plant toxins. The reasons for this replacement are not yet known and await a better understanding of both the ecological and metabolic factors involved. This information is not yet available for adult beetles but, as we will see in the next section, it is starting to emerge for the larvae.

HOST-PLANT INFLUENCE ON LARVAL DEFENSE

The larvae of the subtribe Chrysomelina and of the genus *Phratora* possess nine pairs of segmental exertile glands (Pasteels et al., 1984). Many species produce highly reactive iridoid monoterpene aldehydes and ketones (Table 4). These species feed on a great diversity of host plants (seven families). The plants are not known to contain iridoid precursors, and the iridoid monoterpenes must be synthesized de novo by the larvae. Following the same reasoning as for adult defenses, we suggest that this widespread occurrence of autogenous iridoid monoterpenes represents the primitive condition. Some species, however, produce aromatic compounds such as salicylaldehyde, juglone, and phenylethyl esters, instead of iridoid monoterpenes. The metabolic origin of the phenylethyl esters produced by the North American *Chrysomela interrupta* remains to be clarified (Blum et al., 1972). The other shifts in defensive chemistry can be correlated with host-plant influence. For example, species producing salicylaldehyde feed on *Salix* and *Populus*, which are rich in salicin and from which salicylaldehyde is derived (Rowell-Rahier and Pasteels, 1982; Pasteels et al., 1983; review in Rowell-Rahier and Pasteels, 1986; Pasteels et al., 1989a). Note that in the genus *Phratora*, some species produce autogenous iridoid monoterpenes, but *Phratora vitellinae* derives salicylaldehyde from host-plant salicin, although all species feed on Salicaceae. Thus, as observed in the adults (see above), species belonging to the same genus and feeding on the same host plants may either derive their toxins from host plants or biosynthesize them de novo. The hypotheses advanced to explain this double origin of toxins in the

TABLE 4. DISTRIBUTION OF DEFENSE COMPOUNDS IN CHRYSOMELINAE LARVAE

Beetles	Host plants	Secretions
Subtribe Chrysomelina		
6 genera, 11 spp.	Salicacae Betulaceae Ranuncalaceae Apiaceae Polygonacae Scrophulariaceae Cruciferae	Iridoid monoterpenes
Chrysomela interrupta	Betulaceae	Phenylethyl esters
6 spp. of *Chrysomela*	Salicaceae	Salicylaldehyde ± benzaldehyde
Gastrolina depressa	Juglandaceae	Juglone
Subtribe Phyllodectina		
3 spp. of *Phratora*	Salicaceae	Iridoid monoterpenes
Phratora vitellinae	Salicaceae	Salicylaldehyde

adults are also valid for the larvae. Finally, *Gastrolina depressa* probably derives its juglone (Matsuda and Sugawara, 1980) from the host plant *Juglans*.

To derive salicylaldehyde from salicin, only two enzymes are needed; first, a β-glucosidase to hydrolyze the β-glucoside salicin to the aglycone saligenin and glucose; second, an oxidase to transform saligenin into salicylaldehyde. To derive juglone from the plant toxin, exactly the same two kinds of enzymes are necessary. Likewise, we hypothesized that in the species that secrete iridoid monoterpenes, these chemicals are, initially, produced as glucosides; these are less toxic, more stable, and easier to transfer across membranes (from gland cells to lumen of exertile gland). In the second step, after secretion of the glucosides into the lumen, it is hypothesized that the glycoside is enzymatically hydrolyzed to the hemiacetal and then subsequently oxidized to the dial (e.g., plagodial) (Figure 1). In all these above cases, the common factor is the concurrent action of a β-glucosidase and an oxidase upon a glucoside and an alcohol, respectively, to liberate a carbonyl-containing defensive chemical. Hence, if one views the de novo synthesis of defense chemicals as the primitive state, the enzymes are already (i.e., preadaptively) present that would permit the evolution of the use of plant-derived glucosides for defense. Such an evolution only requires changes in the specificity of the two enzymes, particularly that of the oxidase.

This evolutionary scheme has been tested indirectly (Duffey and Pasteels, in preparation) by comparing the enzymatic specificity of the β-glucosidase and oxidase in the gland fluid of *Plagiodera versicolora*, a species that feeds on

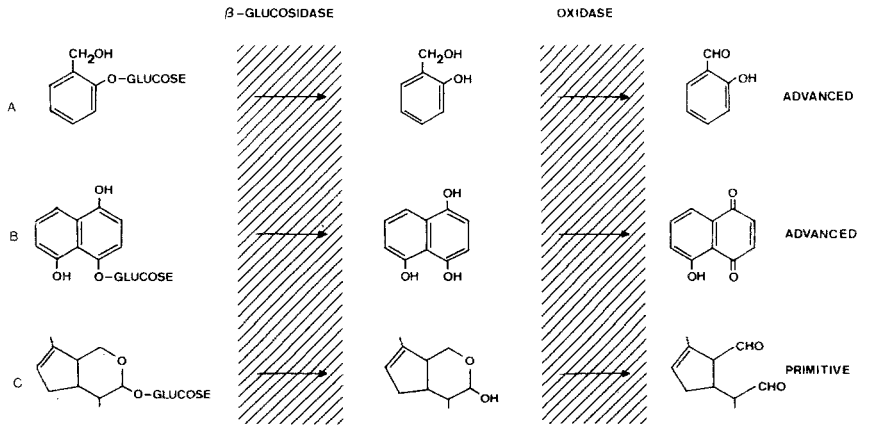

FIG. 1. Final steps in the production of toxins in chrysomelid larvae; A and B examplify the derivation of toxins from plant precursor; C, de novo biosynthesis. B and C remain putative.

Salix and secretes autogenously monoterpenes, and of *Chrysomela populi*, a species that also feeds on *Salix* and by sequestration secretes salicylaldehyde.

Let us consider *Plagiodera versicolora* first; a hypothetical schematic representation of the glands and associated organs and processes involved in the production of iridoidal defenses of larvae is given in Figure 2. The gland cells supposedly biosynthesize and secrete into the lumen of the gland the β-D-glucoside of monoterpene iridoid. Up to now we have not been able to detect the glycosidic or aglyconic hemiacetal form of the monoterpene in the defensive secretion. However, glucose is readily detectable, which supports the hypothesis that a glucoside is a precursor. Moreover, significantly high levels of nonspecific β-glucosidase activity have been detected in this fluid. This activity is able to hydrolyze a wide variety of phenolic and terpenoid glucosides (including salicin). We have not been able to prove the existence of the oxidase, possibly because of its specificity (see below for salicin) and of a lack of appropriate substrates. The secretion was not able to oxidize saligenin to salicylaldehyde or to oxidize the aglycone of the iridoid glucoside mussaenoside.

Another aspect of the scheme deserves attention. The lumen of the gland is filled with a biphasic fluid comprising an aqueous component containing the enzymes and glucose and a nonmiscible phase containing the monoterpene aldehydes. The aqueous phase permits the solution of the postulated glucoside as well as of the aglycone. We assume that once the glucoside is secreted into the

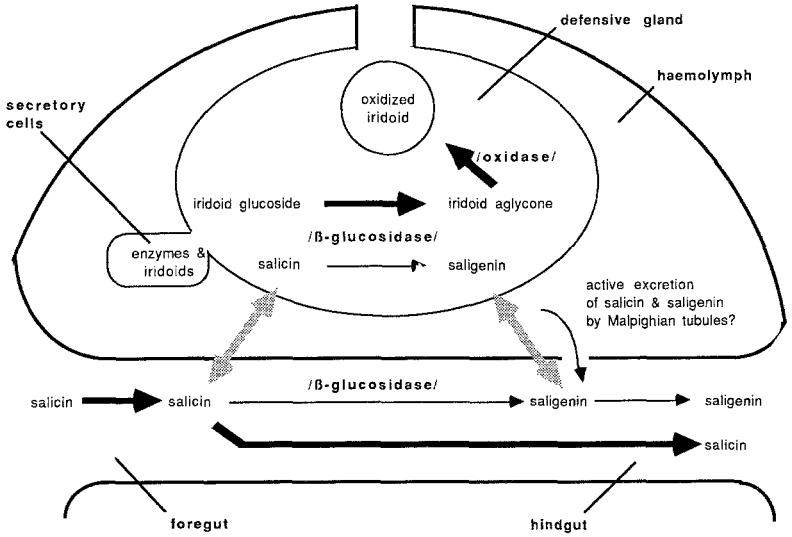

FIG. 2. Schematic representation of the biosynthesis of defensive iridoid monoterpenes in the larvae of *Plagiodera versicolora*. (Adapted from Duffey and Pasteels, in preparation.)

lumen, it is rapidly hydrolyzed, and the resulting aglycone rapidly oxidized to the dial, which is the reason the former two are not detectable. However, once the dial is formed, it rapidly forms the second phase (an oil) because of its immiscibility with the aqueous phase.

Based on the evidence from *Plagiodera versicolora*, we suspect that the major biochemical determinant of the chemical nature of the defensive secretion is the oxidase, rather than the glucosidase. In other words, a change in the specificity of the oxidase, say the acquisition of the ability to oxidize saligenin, would permit an insect to develop a switch from de novo biosynthesis of iridoid monoterpenes to sequestered defense via the utilization of the plant glucoside salicin. In *Chrysomela populi*, we believe we have evidence for this switch in defensive strategy.

In species such as *C. populi*, which produces salicylaldehyde (Figure 3), the defensive secretion is biphasic and readily emulsifiable. The aqueous phase contains not only high levels of both nonspecific β-glucosidase activity (it will hydrolyze many phenolic terpenoid glucosides) and specific oxidase activity (O_2-dependent, NAD-independent activity against a limited number of analogs and homologs of saligenin), but also contains traces of salicin and saligenin (Duffey and Pasteels, in preparation). The determining enzyme, the oxidase, which shows high specificity, rapidly converts saligenin to salicylaldehyde but is unable to oxidize the aglycone of mussaenoside to the aldehydic form; the

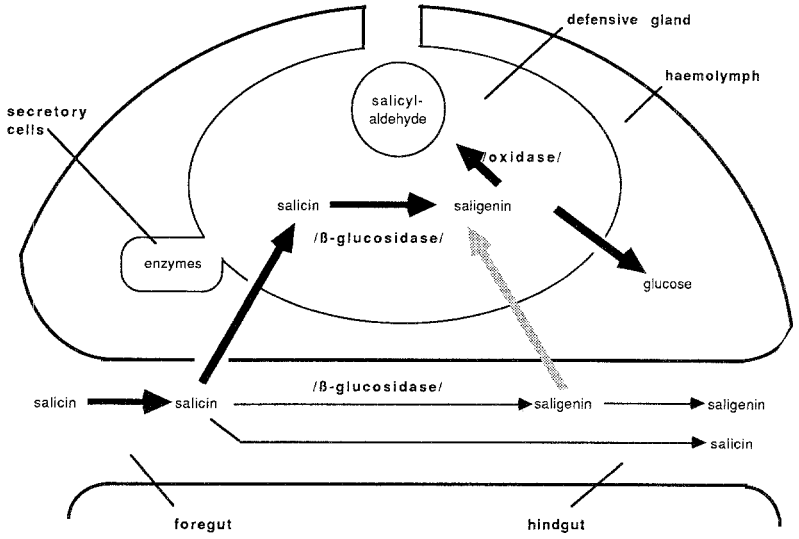

FIG. 3. Schematic representation of the biosynthesis of defensive salicylaldehyde derived from host-plant salicin in the larvae of *Chrysomela populi*. (Adapted from Duffey and Pasteels, in preparation.)

glucosidase, by contrast, effectively hydrolyzes mussaenoside. Once the salicylaldehyde is produced in the gland fluid, it forms a distinct organic phase because of its lipophilic nature and its immiscibility with the aqueous phase. The formation of a water-insoluble phase within the confines of the gland restricts the diffusion of toxic salicylaldehyde to the body in general.

There is another salient aspect of the scheme (Figure 2) that relates to how a change in the specificity of the oxidase could lead to a switch from de novo synthesis of monoterpenes to production of salicylaldehyde from a sequestered precursor. Note again that both *P. versicolora* and *C. populi* feed upon *Salix* and both ingest salicin. Some β-glucosidase activity is present in the gut of *C. populi* and *C. tremulae* (Duffey and Pasteels, in preparation; Pasteels et al., 1983); the ingested foliage does not contribute meaningfully to the production of saligenin. However, only the glands of *C. populi* produce salicylaldehyde. Both salicin and saligenin probably diffuse passively through the bodies of the insects into the gland fluid because of their water solubility. With a change in enzyme specificity towards metabolism of saligenin, resulting from gland glucosidase activity, salicylaldehyde can be accumulated rapidly because of the constant inward diffusion of plant substrates. The conversion of saligenin to salicylaldehyde may also be of benefit, considering that saligenin is toxic and must probably otherwise be eliminated by reglucosylation, excretion by Malpighian tubules, or other mechanisms. It is worth noting that the feces of *C. populi* contain only traces of salicin and saligenin, whereas the feces of adults of *C. populi* and larvae of *P. versicolora*, neither of which sequester salicylaldehyde, have very high levels of salicin and saligenin.

Thus we propose that a single change in the specificity of the oxidase is very likely the only prerequisite to the utilization of plant precursors by the larvae. The process could be further refined by an increased permeability of the gut to salicin and/or saligenin and the reduction of its active excretion by the Malpighian tubules. Additionally, more glucose will be recovered by the larvae as the quantity of salicin hydrolyzed increases, thus giving the species utilizing salicin a net energetic advantage compared to those synthesizing iridoid monoterpenes (Rowell-Rahier and Pasteels, 1986). According to our hypothesis, sequestration of plant-derived toxins is a secondary event that has replaced the more costly autogenous synthesis of toxins.

It is reasonable to suppose that those larvae that secrete juglone underwent an analogous switch in the specificity of the oxidase.

CONCLUSIONS

Our results suggest that during the evolution of leaf-beetles, the colonization of new host plants by these specialized herbivores had major consequences on their chemical defenses. On at least four independent occasions

(once in the adults and three times in the larvae), the sequestration of plant toxins or the derivation of toxins from host-plant precursors has replaced the production of autogenous compounds, although the defensive glands, used both as storage and releasing organs, remained basically unchanged.

Our present understanding of the metabolic processes involved during the derivation of toxins from plant precursors in larval secretions not only illuminates their probable evolution, but also points to the likely limitations of plant influence on larval chemical defense.

Evolutionary success was probably the result of lower defensive costs. Preexisting glands and enzymes (β-glucosidase and oxidase), only slightly modified in their specificity, are used to derive toxins from plants, and thus no additional cost is required for the metabolism of plant precursor by the larvae, and, additionally, there is an economy in toxin biosynthesis and, possibly, in active excretion of the plant toxin. Moreover, the plant toxin acquires a nutritive value due to the glucose released during its metabolism. This is possible only if the plant precursor is a glucoside, the aglycone of which can be transformed by oxidation into a nonpolar toxin. Host-plant influence on larval secretion may be restricted to glucosides. So far, only plant glucosides are known to be used for defense by the larvae, and twice (in *Chrysomela* and *Phratora*) it is the same glucoside, salicin, that is used.

The adult glands must function on completely different principles, which remain poorly understood; plant glucosides are not metabolized in those glands, even in the species that use these glucosides for defense in their larval stages. At present it is impossible even to guess why the sequestration of pyrrolizidine alkaloids is possible in glands that originally biosynthesized cardenolides from cholesterol or why sequestration of pyrrolizidine alkaloids has evolved repeatedly in insects (review in Boppré, 1986).

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A GENERALIST HERBIVORE IN A SPECIALIST MODE
Metabolic, Sequestrative, and Defensive Consequences

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Abstract—Adults of a generalist herbivore, the lubber grasshopper, *Romalea guttata*, can be converted to functional specialists by feeding them exclusively on catnip, *Nepeta cataria*. No obvious adverse effects on adult development resulted from this enforced monophagy. Notwithstanding the fact that *R. guttata* has had no coevolutionary relationship with this Eurasian mint, it readily sequesters compounds that are identical to or derived from the terpenoid lactones that are characteristic of *N. cataria*. *R. guttata* appears to both biomagnify minor allelochemicals and to sequester metabolites of the *Nepeta* terpenes in its paired defensive glands. The levels of autogenously produced phenolics are not affected by feeding on *N. cataria* and the defensive secretions of catnip-fed grasshoppers are more repellent to ants than those of wild-fed acridids. Metabolites of the *N. cataria* monoterpenes are sequestered in the defensive glands when catnip is added to the natural diet of *R. guttata*. The ability of a generalist, *R. guttata*, to facilely bioaccumulate a potpourri of foreign allelochemicals when feeding in a specialist mode is

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analyzed in terms of its biochemical, physiological, and functional significance. Sequestration is examined as a response to the enteric effronteries represented by the phytochemicals that can be characteristic of the "overload" in a monophagous diet.

Key Words—Lubber grasshopper, *Romalea guttata* (*microptera*), Orthoptera, Acrididae, sequestration, catnip, *Nepeta cataria*, cyclopentanoid monoterpenes, phenolics, generalist herbivore, defensive secretion.

INTRODUCTION

Herbivores have evolved a variety of mechanisms for coping with the diverse allelochemicals that fortify their host plants. These include excretion, metabolism, and sequestration (Rothschild, 1972; Blum, 1981, 1983), but the particulars of how a species processes the natural products in its diet are very idiosyncratic and it has been emphasized that each herbivore and the phytochemicals in its host plant must be regarded as a distinct evolutionary case (Blum et al., 1987). Furthermore, the abilities of many species of phytophagous insects to feed on plants containing potentially toxic natural products have considerable consequences vis-à-vis the chemical defenses of these arthropods, especially against vertebrate predators.

Many species of specialist phytophages sequester plant-derived compounds or their metabolites, and these insects are often aposematic (Rothschild, 1972). These acquired compounds are often of considerable defensive value, rendering their possessors either unpalatable or toxic to predators. In addition, these specialists may synthesize intrinsic toxins (Rothschild et al., 1975) that considerably augment the message of deterrency carried by the sequestered allelochemicals. Although some generalists can biomagnify ingested plant natural products, in general these species act like specialists by temporarily feeding on a single plant species that often contains high concentrations of sequestrable compounds (Rothschild and Aplin, 1971).

While most specialist herbivores sequester plant allelochemicals in nonsecretory structures (Duffey, 1980), species in a few taxa bioaccumulate these compounds in exocrine glands, some of which are identified with the de novo synthesis of potent defensive allomones. Lygaeids may biomagnify cardenolides in their aldehyde-rich defensive glands (Scudder and Duffey, 1972; Duffey and Scudder, 1974), in contrast to certain milkweed-feeding acridids whose defensive exudates are dominated by these steroidal allelochemicals (von Euw et al., 1967). The generalist acridid, *Romalea guttata*, appears to represent a unique case because it not only produces a large variety of allomonal constituents in its defensive glands (Eisner et al., 1971; Jones et al., 1988), but when forced to feed on a single species of host plant behaves like a sequestrative

specialist (Jones et al., 1989). The consequences of this enforced monophagy are considerable.

The secretions of *R. guttata* are dominated by phenolics and quinones (Eisner et al., 1971), but great individual variation in the compositions of the secretions are characteristic of this species (Jones et al., 1986). Furthermore, the autogenous defensive compounds in the secretions may be quantitatively and qualitatively diminished when diet breadth is restricted (Jones et al., 1987). Significantly, when diet was reduced to a single species, wild onion (*Allium canadense*), allelochemicals characteristic of this plant were sequestered in the defensive glands, whereas autogenous compounds were either absent or present in reduced concentrations (Jones et al., 1989). However, the secretions of the onion-fed acridids were more repellent to foraging ants than those derived from wild-fed grasshoppers.

Recently, we demonstrated that adults of *R. guttata* fed readily on catnip, *Nepeta cataria*, as a sole food plant, and apparently sequestered some compounds from this mint (Blum et al., 1987). In the present report, we have determined the fates of the ingested monoterpenes characteristic of *N. cataria* (Regnier et al., 1967a,b; Tucker and Tucker, 1988), when *R. guttata* was in a specialist feeding mode. In addition, we studied the effect that this enforced monophagy had on the concentrations of autogenous defensive compounds and whether this dietary specialization affected adult survivorship. Finally, we compared the repellency of the secretions from *Nepeta*-fed acridids to those collected from wild-fed individuals as a further measure of the consequences of monophagy for this eminent polyphage.

METHODS AND MATERIALS

Rearing of Insects. Groups of 15 field-collected female larvae (fifth instar) were placed in cages maintained at 25 ± 2°C with 60 ± 10% relative humidity and a 12 : 12 hr light–dark cycle. Larvae and the subsequent adults were either fed a control diet consisting of romaine lettuce, *Latuca sativa longifolia*, and dried oatmeal or experimental diets made up of (1) freshly cut leaves and stems of catnip, (2) a natural diet (wild fed) that included many of the 26 species of plants readily fed upon in this area, and (3) natural diet plus catnip as a minor constituent. Survivorship was recorded 14 days after the adult molt.

Secretion Collection. The defensive exudate is eliminated at each molt and subsequently regenerated. Two-day-old adults were milked with microcapillaries (10 μl), and these secretions were discarded. Secretions were again collected when the adults were 14 days old, and the total volume of secretion was recorded (±0.25 μl). The capillaries were immediately sealed and were stored at −10°C until the secretions were analyzed.

Bioassay of Romalea *Secretions as Feeding Deterrents.* The repellencies of the metathoracic exudates were determined by utilizing the fire ant *Solenopsis invicta* in feeding tests. Bioassays were undertaken by introducing into the foraging arena of colonies ($N = 8$) 25 × 75-mm glass slides to which were added two 25-μl droplets of 2.5% sucrose solution. One droplet contained 0.5 μl of a secretion from lubbers that had been fed on a single host plant (e.g., lettuce, catnip). The second droplet served as a standard and contained 0.5 μl of secretion obtained from wild lubbers that had consumed a generalist diet (Jones et al., 1987). Tests were undertaken by presenting the different secretions in a randomized order and at different times of the day. The number of ants at each droplet was recorded at 5-min intervals until a droplet had been consumed. Data were analyzed by Friedman two-way analysis of variance.

Analysis of Defensive Exudates, Feces, and Catnip Leaves. Fresh catnip leaves and feces from grasshoppers that had fed exclusively on catnip were analyzed for the presence of *Nepeta*-derived compounds or their metabolites. Leaf or fecal samples were placed in 3-dram vials and covered with methylene chloride (Burdick and Jackson) and stored at −10°C for at least 30 days. Prior to examination by gas chromatography (GC) or gas chromatography–mass spectrometry (GC-MS), the samples were warmed to room temperature, shaken, and a 250-μl aliquot in a tapered test tube was concentrated under nitrogen to 5 μl. The extract was treated with 100 μl of a 1:1 mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and dimethylformamide (DMF), capped, heated for 45 min at 76°C in a capped tapered test tube and, after cooling, a 1-μl aliquot was removed for gas chromatographic (GC) analysis. Derivatization of compounds in the defensive secretions was effected by adding the BSTFA–DMF mixture to a 5-μl aliquot of the exudate in a microsampling vial, which was treated as above.

GC and GC-MS analyses were conducted on a Hewlett-Packard 5985B GC-MS system equipped with a cold on-column injection port (Arrendale and Chortyk, 1985) with a 0.32-mm-diam. × 30-m SE-54 0.5-μm film thickness fused silica capillary column (Arrendale and Martin, 1988), and a flame ionization detector (FID) and an open split GC-MS interface (Arrendale et al., 1984) using a temperature program of 100°C for 1 min followed by 3°C/min increase to 280°C. Samples were first analyzed with the column connected to the FID and then switched to the open-split interface for GC-MS analysis. Mass spectrometer conditions were as follows: ion source temperature 200°C, electron multiplier voltage 2400 V, electron energy 70 eV, scan range 40–400 dalton, scan rate 266.7 dalton/sec, and GC-MS interface zone temperature 300°C.

Synthesis of Nepetalactone Metabolites. Potential metabolites of nepetalactone (**Ia,** Figure 1) were prepared by isolating the lactone from *N. cataria.* Leaves and small stems (285 g) were extracted by dipping into 200 ml of methylene chloride and, after filtration and solvent removal, a 2.5-g cuticular extract

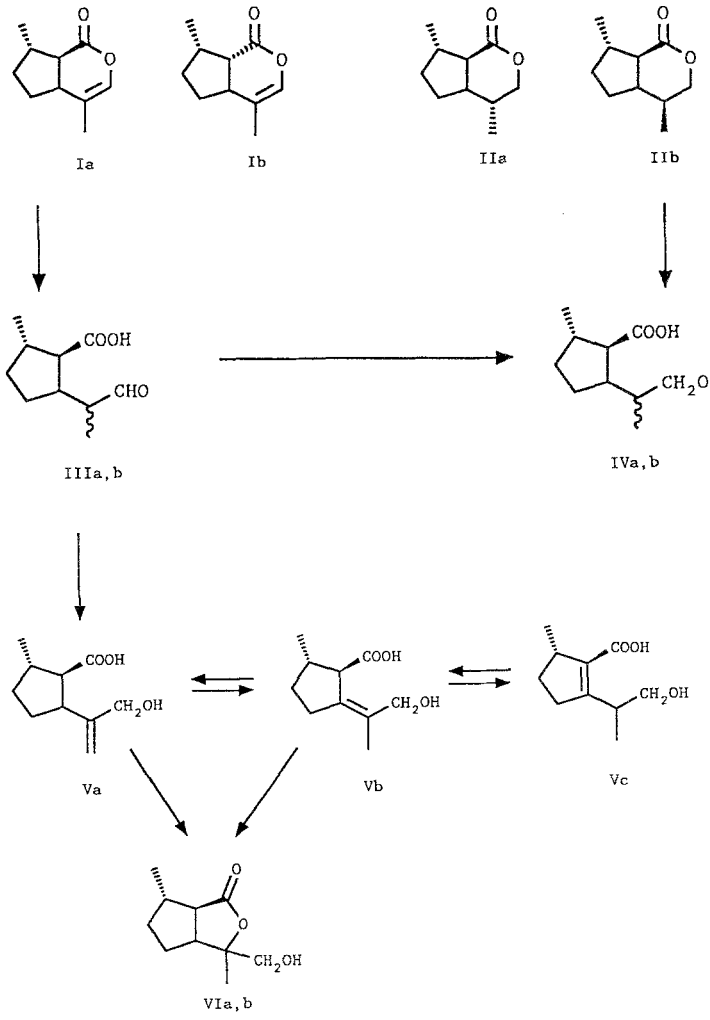

FIG. 1. Cyclopentanoid monoterpenes from *N. cataria* and their metabolites in lubber secretions.

was obtained. A portion of the extract (0.85 g in hexane) was placed on a 30-g silicic acid column (Unisil, 100–300 mesh, activity grade I) and eluted stepwise with hexane–CH_2Cl_2 gradient (100 ml hexane; 200 ml, 1:9 CH_2Cl_2–hexane; 200 ml, 1:4 CH_2Cl_2–hexane; and 200 ml, 1:1 CH_2Cl_2–hexane). About 122 mg of 93+% I was isolated in the 1:1 CH_2Cl_2–hexane fraction.

A portion of the isolate was subjected to preparative GC on HP 5830

equipped with a 2-mm-ID × 1.8-m glass column packed with 10% Carbowax 20 M on 80–100 mesh Chromosorb W-AW and thermoconductivity detector using a temperature program of 100–200°C at 5°C/min to yield 98% Ia.

*Conversion of **Ia** to Dihydronepatalacone (**IIa**) and Isodihydronepatalactone (**IIb**).* About 1.0 mg of **Ia** was hydrogenated using palladium on charcoal in 95% ETOH under H_2 at atmospheric pressure. A mixture of products similar to those isolated by Regnier et al. (1967b) was obtained: 82% 2-isopropyl-5-methylcyclopentanoic acid, 2% **IIa** (Figure 1, MW = 168), and 16% **IIb** (Figure 1, MW = 168).

*Conversion of **Ia** to Nepetalic Acids (**IIIa** and **b**) and Nepetolic Acids [**IVa** and **b** = 2-(2-Hydroxyisopropyl)-5-methylcyclopentanoic Acids].* About 1 mg of **Ia** (Figure 1) was hydrolyzed with 1.5 ml of 1 N KOH for 1 hr in an ultrasonic bath (Regnier et al., 1967a), and the hydrolysate was extracted with hexane (3 × 1 ml). Half the water-soluble portion was adjusted to pH 2 and was extracted with CH_2Cl_2 (3 × 1 ml) to yield a mixture of **IIIa** and **b,** which was converted to silyl ethers and subjected to GC and GC-MS analysis (MW_{TMS} = 256). The nepetalic acids were not observed in the defensive secretions of *R. guttata*.

The other portion of the **Ia** hydrolysate was directly treated with excess $NaBH_4$. After 24 hr in the dark at room temperature, the sample was treated as described above to yield ditrimethylsilyl derivatives of dihydroisonepetolic acid (**IVa,** MW = 330) and dihydronepetolic acid (**IVb,** MW = 330) in about a 1:2 ratio. Hydrolysis of the hydrogenation products (**IIIa** and **b**) from **Ia** produced about a 1:6 ratio of **IVa** to **IVb.**

RESULTS

7-Methylcyclopentapyranones and Other Phytochemicals in N. cataria *Leaves.* The capillary gas chromatogram of the CH_2Cl_2 whole-leaf extract of catnip is shown in Figure 2. The majority of the compounds eluting before 12 min are artifacts of the silylation reagents. In good agreement with other reports (Regnier et al., 1967a,b), the terpenoid lactones associated with the extract were 92% **Ia** (nepetalactone), 7.4% **Ib** (epinepetalactone), 0.1% **IIa** (dihydronepatalactone), and 0.3% **IIb** (isodihydronepatalactone) (see Figure 1 for structures).

Ib was characterized using retention time and GC-MS data obtained from the analysis of an essential oil sample from *Nepeta mussini*; the major terpene in this oil is **Ib** (Regnier et al., 1967b). The presence of the saturated lactones **IIa** and **IIb** was confirmed by hydrogenation of **Ia** and reported GC retention data (Regnier et al., 1967a,b).

Other components identified by GC-MS were the typical plant fatty acids

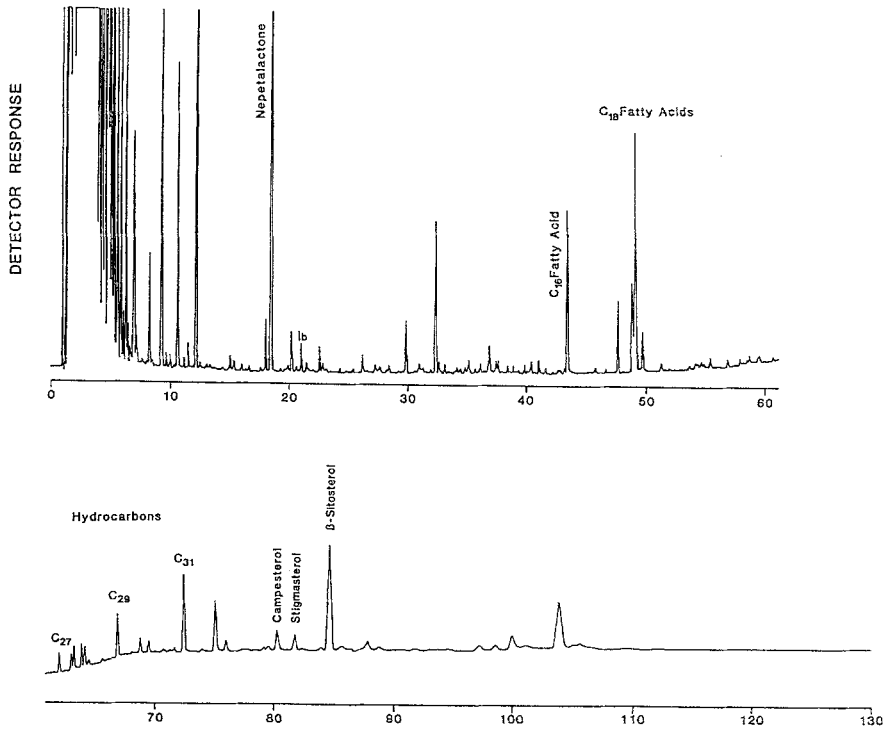

FIG. 2. Capillary gas chromatogram of the silylated methylene chloride extract of *N. cataria* leaves.

and a series of aliphatic hydrocarbons dominated by the C_{27}, C_{29}, and C_{31} alkanes (Figure 2). Typical phytosterols were also identified in this extract. None of the phenolics (e.g., catechol, hydroquinone) that dominate the defensive secretion of *R. guttata* were detected.

Phytochemicals Identified in Feces of Lubbers Fed Leaves of N. cataria. Gas chromatographic analysis of the fecal extracts of catnip-fed lubbers (Figure 3) indicated that most of the hydrocarbons, fatty acids, and phytosterols were excreted directly after ingestion of leaf material. On the other hand, major qualitative and quantitative changes in the cyclopentanoid monoterpenes of *N. cataria* were readily evident.

The major terpene present in catnip leaves **Ia,** was reduced from a relative abundance of more than 90% of the monoterpene mixture to a relative abundance of less than 20% in the fecal extract (Figure 3). Indeed, none of the five terpenes detected in the feces exceeded 25% of the monoterpene mixture and both the unsaturated (**Ia, Ib**) and saturated lactones (**IIa, IIb**) were of similar

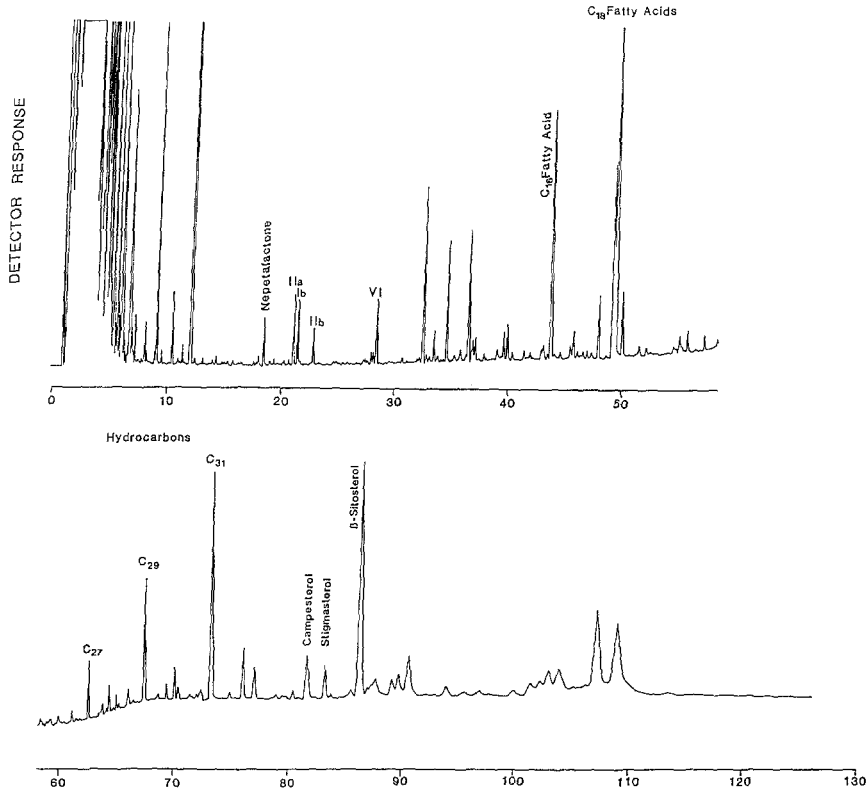

FIG. 3. Capillary gas chromatogram of the silylated methylene chloride extract of the feces of lubbers fed on *N. cataria* leaves.

concentrations. On the other hand, a terpene not detected in *N. cataria* leaves was present in the feces of catnip-fed grasshoppers. This lactone, **VI,** is described in the next section.

Neither the characteristic cyclopentanoid monoterpenes of *N. cataria* nor their metabolites are excreted in appreciable quantities. For example, based on unitary detector response, the ratio of **Ia** and the C_{31} cuticular hydrocarbon in the leaf was 176:1, whereas the ratio in the feces was 1:5.

Compounds Identified in Secretions of Lubbers Fed N. cataria. The capillary gas chromatogram of the defensive secretion (TMS) of lubbers fed on catnip is shown in Figure 4. The major compounds detected by GC-MS appeared to be a mixture of silylated hexoses that were present in all exudates analyzed, host plants notwithstanding. The structures of these compounds were not further characterized.

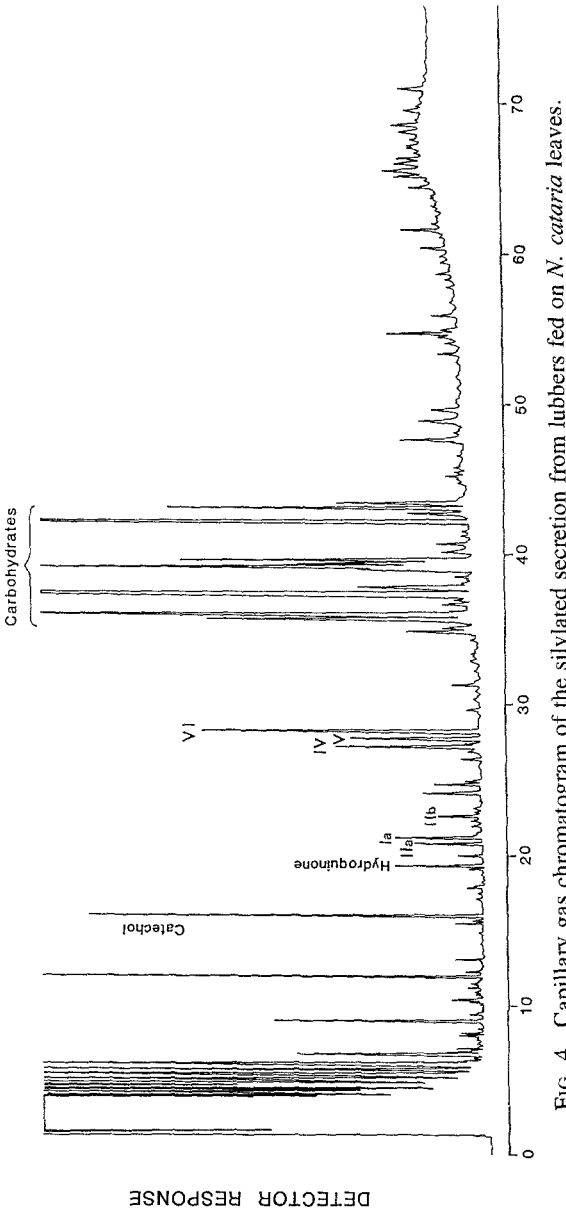

FIG. 4. Capillary gas chromatogram of the silylated secretion from lubbers fed on *N. cataria* leaves.

Two of the phenolics characteristic of lubber defensive exudates, catechol, and hydroquinone, were consistently identified in the lubber defensive secretions (Figure 4). For adult lubbers fed solely on *N. cataria*, the catechol–hydroquinone ratio was about 5 : 1, as was the case for the secretions of grasshoppers that had consumed a natural diet (wild fed). Approximately the same ratio was present in secretions of lubbers that had been fed an extract of *N. mussini* applied to oatmeal. When *R. guttata* adults were fed their natural diet plus about 10% catnip leaves, the ratio of these two phenolics increased to approximately 20 : 1. On the other hand, the ratio of catechol–hydroquinone varied from 1 : 4 to 1 : 10 when the acridids were fed a control diet of lettuce leaves and oatmeal.

Six monoterpenes were identified in the defensive exudates of catnip-fed adults, three of which consisted of minor compounds present in the catnip essential oil. **Ib, IIa,** and **IIb** were biomagnified in the secretion, increasing from about 10% of the monoterpenes in the leaf to about 25% (Figure 4). A comparison of the mass spectral data for synthetic **IIa** and **IIb** with those found in the exudate is presented in Figures 5 and 6. The mass spectrum of **Ib** from the defensive secretion is shown in Figure 7. Although **Ia,** the major terpene produced by *N. cataria* (Figure 2) was not detected, three metabolites of this lactone, designated **IVb, V,** and **VI,** which constituted about 75% of the detected terpenes, were found.

The basic structure of these hydroxy-acids, **IV** (Figure 1), consisting of about 20% of the terpenoid mixture, was confirmed by the hydrolysis of **Ia** to a mixture of nepetalic acids, **IIIa** and **b,** and reduction with sodium borohydride to yield a mixture of nepetalic acids **IVa** and **b** (Figure 1). They were also prepared by the hydrolysis of the mixture of reduced lactones **IIa** and **IIb**. Only one isomer of **IV** was observed in the secretion. Based on relative abundance of synthetic products and relative retention data, the compound in the secretion was assumed to be dihydronepatalic acid. The mass spectrum of the ditrimethylsilyl derivative of the synthetic compound and the compound in the exudate are compared in Figure 8.

The mass spectrum of secretory component **V,** also representing about 20% of the terpenoid mixture, is shown in Figure 9. The molecular weight (328) and fragment ions at *m/z* 73 and 147 indicated that two methylsilyl groups were present (Draffan et al., 1968), suggesting that **V** was an isomerization product of **III** (Figure 1). Three probable structures for this compound, the methylhydroxyisopropenylcyclopentanoic acid (**Va**), the methylhydroxyisopropylidenecyclopentanoic acid (**Vb**), and the methylhydroxyisopropylcyclopenetenoic acid (**Vc**), could result.

Based on the MS fragmentation pattern (Figure 10), the most probable structure for **V** is **Vb** (Figure 1). Abstraction of the proton from the β-carbon of the cyclopentane ring would result in the *cis* elimination of the trimethylsilyl

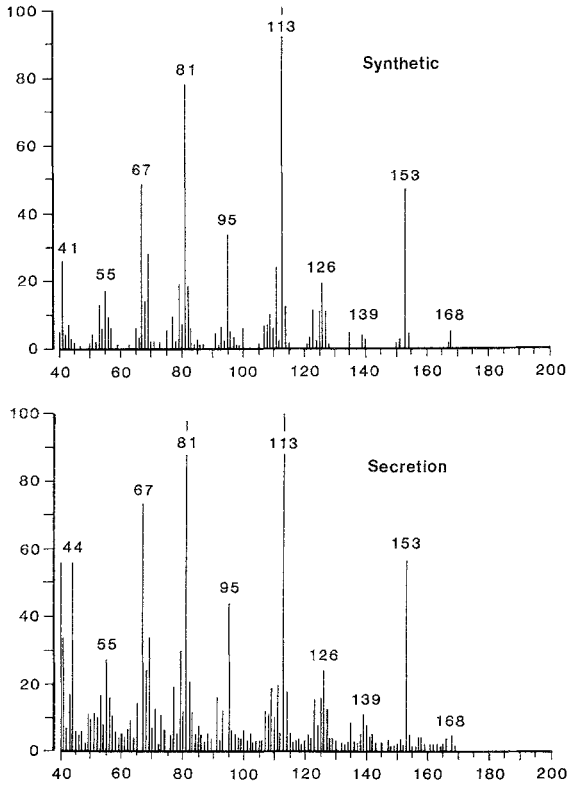

FIG. 5. Comparison of the mass spectra of synthetic dihydroisonepetalactone (**IIa**) and **IIa** from the secretions of lubbers fed on *N. cataria* leaves.

ether of formic acid and the formation of the very stable ion at *m/z* 210, which is the second most abundant ion in the mass spectrum (Figure 9). A similar loss from the M-15 ion or the loss of a methyl radical from the 210 ion would yield the third most abundant ion of *m/z* 195. Abstraction of the active proton alpha to the carboxyl group and double bond by the silyl ether moiety would eliminate the HOTMS and produce the stable ion at *m/z* 238.

The mass spectrum of compound **V**, the major terpene in the secretion (≈40%), is shown in Figure 11. The proposed structure can be formed from **V**, and **VI** clearly fits the mass spectral data. All major fragment ions are explained by the proposed fragmentation scheme (Figure 12). The key to the characterization of this compound was the base peak at *m/z* 153.

Metabolites **IV**, **V**, and **VI** are sequestered in the defensive glands of lub-

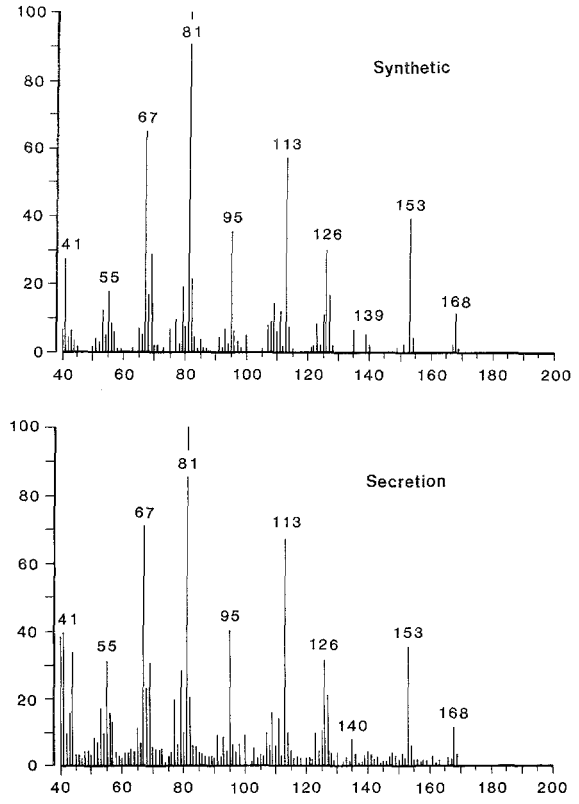

FIG. 6. Comparison of the mass spectra of synthetic dihydronepetalactone (**IIb**) and **IIb** from the secretions of lubbers fed on *N. cataria* leaves.

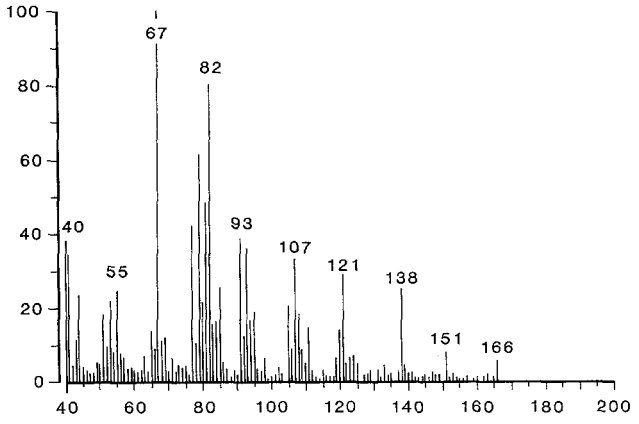

FIG. 7. The mass spectrum of epinepetalactone (**Ib**) from the secretion of lubbers fed on *N. cataria*.

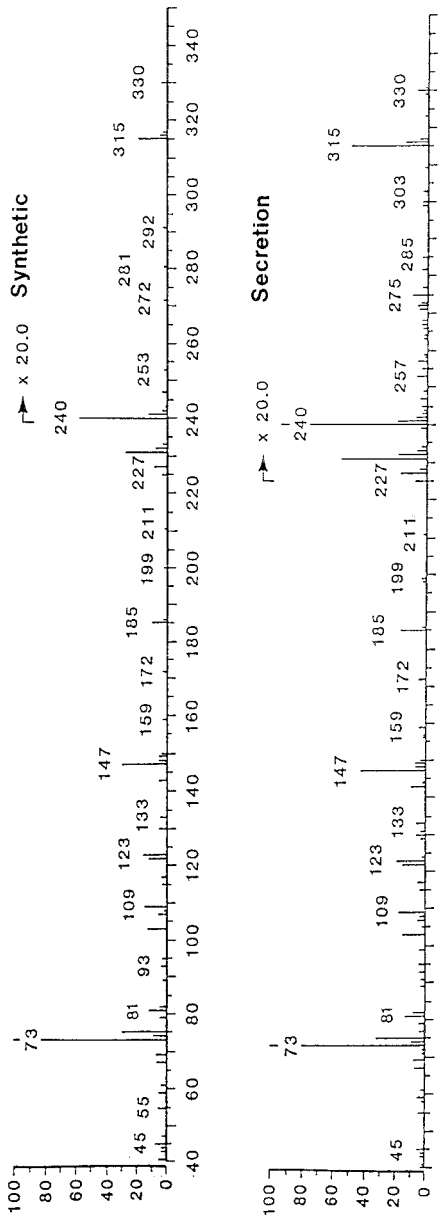

FIG. 8. Comparison of the mass spectra of the ditrimethylsilyl derivatives of synthetic dihydronepatalic acid (**IV**) and **IV** from the secretions of lubbers fed on *N. cataria* leaves.

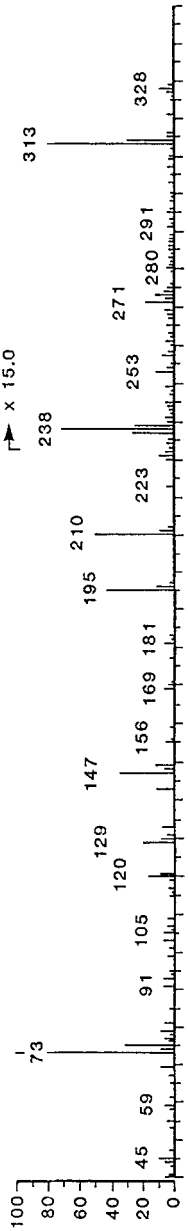

FIG. 9. The mass spectrum of the ditrimethylsilyl derivative (V) from the secretion of the lubbers fed on *N. cataria* leaves.

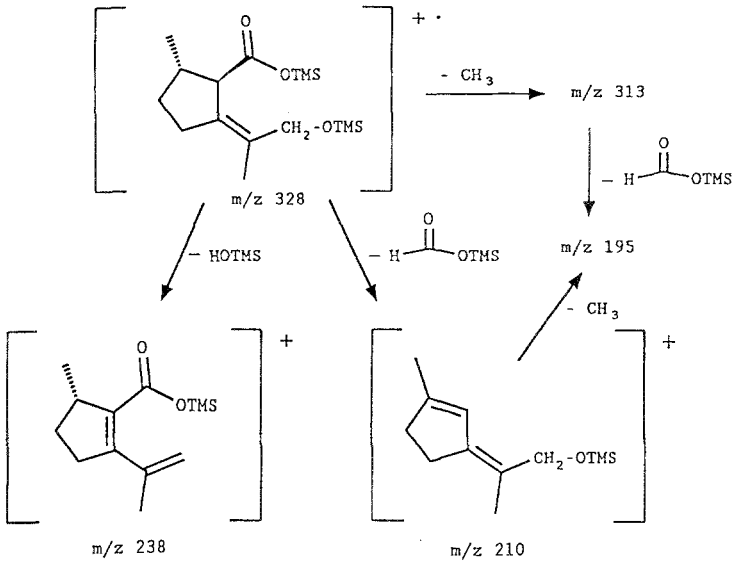

FIG. 10. The mass spectral fragmentation of the ditrimethylsilyl derivative of **V**.

bers that had fed on a natural diet to which was added catnip leaves. As was the case for lubbers that had fed exclusively on catnip as adults, compound **VI** was the major terpenoid present. On the other hand, the concentrations of the three terpene metabolites were considerably lower in these secretions than those of grasshoppers that had specialized on *N. cataria*.

Efficacies of Defensive Secretions as Feeding Deterrents. Secretions from lubbers that had fed exclusively on catnip were very repellent to hungry ant workers. Utilizing solutions fortified with secretions from wild lubbers that had fed on a natural diet as a standard, it was determined that exudates from catnip-fed acridids were about three times as repellent. In comparison, secretions from lettuce- and oatmeal-reared acridids were about two thirds as repellent as the standard. Among secretions bioassayed from lubbers fed on 13 single-host diets, only that of wild carrot, *Daucus carota*, was of approximately equivalent repellency to that of catnip both in terms of ant repellency and time taken for the sucrose-fortified droplets to be eaten (unpublished data).

Survivorship and Secretory Volumes of Lubbers Fed on Different Diets. Larvae and adults of *R. guttata* fed as readily on catnip leaves as they did on those of the natural generalist diet. No significant difference in 14-day mortality resulted in adults that had fed exclusively on catnip compared to the control diet of lettuce plus oatmeal or the natural polyphagous diet. Thus, as far as we

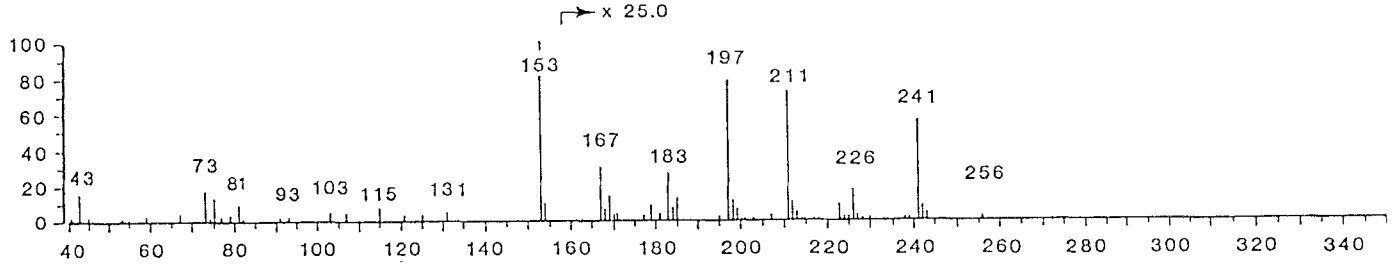

FIG. 11. The mass spectrum of the trimethylsilyl derivative (**VI**) from the secretion of the lubbers fed on *N. cataria* leaves.

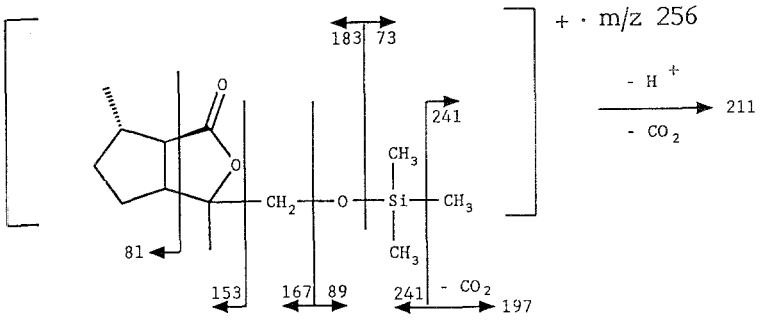

FIG. 12. The mass spectral fragmentation of the trimethylsilyl derivative of **VI.**

can determine, *N. cataria* is an eminently acceptable host plant for the developing adults.

We have found that there is a positive correlation between the amount of secretion generated by adult *R. guttata* and fitness characteristics such as survivorship, weight, and rate of development (Whitman, Jones, and Blum, unpublished data). Ingestion of catnip as the sole host plant resulted in the highest average secretion obtained with lubbers that had fed on 13 different diets. Individuals that had fed exclusively on catnip produced an average of 4.5 μl secretion/individual, whereas control grasshoppers that ingested solely lettuce and oatmeal produced an average of about 2 μl of secretion each. Indeed, only grasshoppers that had utilized tansy, *Tanacetum vulgare*, as their sole food plant produced secretions that averaged more than 4 μl/individual. For the 13 single host-plant diets, an average of 2 μl of secretion/individual was obtained.

DISCUSSION

R. guttata is an eminent generalist that considerably enhances its polyphagous propensity by exhibiting obligate host-plant switching that includes feeding on different plant tissues of different ages. By nature a nibbler, this acridid samples many of the plant species in its habitat, switching frequently from one to another, as is the case for some other grasshopper species (Waldbauer and Friedman, 1988). We suggest that this obligate switching of host plants is an adaptation that results in reducing the physiological stress that could occur from the ingestion of inordinately high levels of the characteristic allelochemicals produced by a single species of host plant. On the other hand, it is possible that the pronounced polyphagy of *R. guttata* may reflect selection for a diet that is optimal for rapid growth and development as well as fitness (Waldbauer and Friedman, 1988). However, even if we do not comprehend the *raison*

d'être for the pronounced polyphagy of this acridid, we recognize that the results of its idiosyncratic feeding behavior may affect its survivorship.

The defensive efficacies of the metathoracic secretions of *R. guttata* may be significantly correlated with diet breadth. The secretions produced by lubbers feeding on a diet consisting of lettuce or an artificial diet lacking fresh plant material were less repellent to ants than those derived from acridids reared on a natural polyphagous diet. Furthermore, the concentrations of phenolics in exudates produced by lubbers reared on the above restricted diets were reduced compared to exudates from lubbers reared on a natural generalist diet (Jones et al., 1989). However, the reduction in autogenous phenolics observed with secretions from onion-reared lubbers was accompanied by the sequestration of low concentrations of onion-derived sulfides with a concurrent increase in the deterrency of the exudates so that they were more repellent than those generated by lubbers on a natural wild diet. Similarly, lubbers feeding solely on catnip sequestered compounds derived from this mint, resulting in an increase in ant deterrency of the exudate. However, the secretory and sequestrative specifics of catnip monophagy differ strikingly from those observed with wild onion.

Specialization on onion results in secretions that contain sulfur volatiles with a concentration about 300-fold less than the natural phenolics, whose mean concentrations were about 37 times lower than those from exudates of wild-fed lubbers (Jones et al., 1989). By contrast, secretions of lubbers specializing on catnip did not contain reduced levels of phenolics such as catechol and hydroquinone. Furthermore, the combined concentrations of the sequestered monoterpenes were about a third greater than the two phenolics (Figure 4). Therefore, the increase in deterrency of the secretions from lubbers specializing on catnip may reflect the added repellent impact of high levels of the mint-derived phytochemicals in combination with the normal levels of proven deterrents, the autogenous phenolics. Thus, reduction in diet breadth, as exemplified by *N. cataria*, does not appear to depress the concentrations of autogenous defensive compounds in lubber secretions, in contrast to what has been observed for secretions produced by lubbers reared on an artificial diet or another single-host plant, wild onion (Jones et al., 1987, 1989). Since catnip and wild onion appear to differ considerably in their effect on autogenous phenolics in defensive secretions, it seems appropriate to determine how sensitive the concentrations of these compounds (i.e., catechol, hydroquinone) are to enforced monophagy in other cases. Beyond that it is appropriate to ask how important these phenolics are to the observed repellencies of the defensive exudates.

The ratio of catechol to hydroquinone in secretions of lubbers feeding solely on catnip was about 5 : 1, as was the case for these compounds in the exudates of lubbers that had fed on a natural polyphagous diet. Since the concentrations of these two phenolics are similar in both secretions, the fact that the secretions of catnip-fed lubbers are about three times more repellent to ants as those derived

from insects fed on natural diets must reflect the presence of compounds other than catechol and hydroquinone in the former exudates.

We have also noted that in the case of secretions produced by lubbers feeding on a control diet of lettuce and oatmeal, the phenolic ratio was about 1:7, with hydroquinone predominating. This secretion is considerably less repellent than the wild diet. In the case of a single-host plant diet consisting of garlic, *Allum sativum*, a relative of wild onion, neither catechol nor hydroquinone could be detected in the defensive exudate. The defensive efficacy of these secretions was very similar to that of lettuce and oatmeal. It thus appears that both the ratio and concentration of autogenously derived phenolics may affect the repellencies of the defensive secretions of lubbers feeding on different diets.

The ability of diet to affect the concentrations of catechol and hydroquinone in the metathoracic exudates raises the question of the origin of these so-called autogenous defensive compounds. Adults of *R. guttata* produce low concentrations of these two phenolics when they are reared on an artificial diet lacking polyphenolic compounds that could serve as precursors (Jones et al., 1987). On the other hand, a portion of the catechol and hydroquinone could well be derived from aromatic amino acids (e.g., tyrosine), as are phenolics produced in the defensive secretions of millipedes (Duffey and Blum, 1977). By contrast, the ability of diverse host plants to effect the levels of these two phenolics in the secretions makes it seem probable that phytochemicals such as polyphenolics (e.g., chlorogenic acid) may also serve as precursors of catechol and hydroquinone. Thus, these autogenously produced defensive allomones appear to have two unrelated origins, one of which (e.g., phytochemicals) may be of much greater significance than the other. Indeed, if catechol and hydroquinone are each administered as 10% solutions on oatmeal along with lettuce, the resulting defensive secretions contain abnormally high levels of the administered phenolics. Obviously, ingestion of free phenolics in plant tissues and their subsequent sequestration in the defensive secretions could serve admirably to increase their concentrations in the exudates.

Phenolic concentrations are also drastically affected when catnip is added to the natural polyphagous diet. The catechol–hydroquinone ratio of the secretions of acridids feeding on a catnip-fortified natural diet was about 20:1, in contrast to the 5:1 ratio that prevails when catnip was the sole food plant. In addition, lubbers sequester terpenes in their defensive exudates when feeding in a generalist mode that includes catnip, thus demonstrating that biomagnification can occur even in a generalist that is ingesting a polyphagous diet. No evidence for allelochemical sequestration when feeding in a generalist mode was detectable when adults of *R. guttata* fed on a natural diet that included sulfide-rich wild onion (Jones et al., 1989).

Although selected generalist herbivores can sequester allelochemicals (Rothschild and Aplin, 1971), these phytophages are feeding on a single host

plant when this occurs, and thus are functional specialists. Conceivably, *R. guttata*, a species of low vagility, may manifest temporary specialization as new species of host plants become available as a consequence of plant successional changes that occur in its limited habitat during its two- to three-month developmental period. Such temporary specialization could serve to fortify its defensive secretions with additional defensive allomones. The nepetalactone metabolites in the exudates of lubbers feeding on a polyphagous diet including catnip are at a low concentration relative to their levels in secretions derived from grasshoppers feeding only on catnip. Although increased diet breadth appears to result in dilution of sequestered plant natural products (Jones et al., 1989), fortification of the exudates with catnip metabolites undoubtedly provides them with an increased defensive "punch."

The sequestration of specific terpenoids by *R. guttata* emphasizes that insects can be highly selective in terms of the allelochemicals that are biomagnified. Nepetalactone, the major monoterpene lactone produced by *N. cataria*, does not appear to be sequestered, whereas three minor compounds constituting about 10% of the available lactones, are biomagnified so that they represent 25% of the stored terpenoids. Significantly, about 75% of the sequestered monoterpenes are degradation products of the lactones produced by *N. cataria*. About 50% of the stored metabolites are made up of nepatolic and nepetolic acids (Figure 1), whereas a novel lactonic metabolite constitutes the remaining half of the sequestered compounds. The sequestrative strategy of *R. guttata* is similar to that of arctiid species that biomagnify some minor allelochemicals while metabolizing others that are subsequently stored (Aplin and Rothschild, 1972). While the sequestrative fingerprint of *R. guttata* may be correlated with the physical properties (e.g., lipophilicity) of the compounds, it is important to recognize that some biomagnified compounds (e.g., metabolites) may also represent true detoxification products. In essence, the processing of these allelochemicals by the lubber, while unpredictable, may ultimately be demonstrated to reflect the ability of this insect to both sequester some compounds directly from the plant and to detoxify others to metabolites that can be facilely biomagnified.

The great efficiency of *R. guttata* as a sequestrator of the cyclopentanoid monoterpenes of catnip is evidenced by their low concentrations in feces (Figure 2). For example, the major terpene produced by catnip, nepetalactone, which is not sequestered in the defensive secretion, is excreted in the feces as a very minor constituent. Other catnip terpenes such as isodihydronepetalactone and dihydronepetalactone, which constitute about 2% of the leaf monoterpenes (Figure 2), represent nearly 40% of the fecal terpenoids (Figure 3). The major terpene metabolite in the defensive secretion is also detectable in the feces, but in considerably reduced concentration. In contrast, the leaf cuticular hydrocarbons, sterols, and fatty acids (Figure 2) appear to have been excreted in con-

centrated form (Figure 3). It thus appears that *R. guttata*, in common with arctiid larvae (Aplin and Rothschild, 1972), is a very efficient—and selective—sequestrator of allelochemicals.

Finally, the ability of this North American acridid to readily sequester the cyclopentanoid monoterpenes of an Eurasian plant, *N. cataria*, with which it has had no coevolutionary relationship, raises the question of how selective sequestration is vis-à-vis allelochemicals. Duffey (1980) has emphasized that sequestration is a common phenomenon in herbivores, encompassing a multitude of compounds belonging to diverse chemical classes. We have found that the secretions of lubbers fed single host plants are often enriched with a variety of nonautogenous compounds and that these exudates are often considerably more repellent than those from wild-fed grasshoppers. In short, the pronounced mithridatism of *R. guttata* often results in the sequestration of a variety of plant natural products, or their metabolites, with a concomitant increase in defensive efficacy of the exudates. Our results suggest that sequestration of specific allelochemicals may not reflect any previous adaptation on the part of the herbivore, but rather a concatenation of biophysical and biochemical processes that follows the ingestion of intrusive natural products. Ultimately, the sequestration of plant-defensive compounds by herbivores may be explained as the inevitable consequence of a herbivorous life-style associated with the pharmacopoeia represented by the host-plant arsenals.

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WATERCRESS AND AMPHIPODS
Potential Chemical Defense in a Spring Stream Macrophyte

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Abstract—We investigated the potential role of defensive chemicals in the avoidance of watercress (*Nasturtium officinale*) by the cooccurring amphipod, *Gammarus pseudolimnaeus* at two spring brooks: Carp Creek, Michigan and Squabble Brook, Connecticut. We conducted observations and laboratory experiments on the consumption of watercress, the toxicity of damaged (frozen) watercress, and the toxicity of damage-released secondary chemicals. Field-collected yellowed watercress typically lacked the bite and odor characteristic of green watercress and was consumed by *G. pseudolimnaeus*. *G. pseudolimnaeus* strongly preferred yellowed watercress to green watercress despite the higher nitrogen content of the latter (2.7 vs 5.4%), and usually consumed five times more yellowed watercress (>50% of yellowed leaf area vs. <8% of green leaf area presented). Fresh green watercress contained seven times more phenylethyl glucosinolate than yellowed watercress (8.9 mg/g wet vs. 1.2 mg/g). Cell-damaged (frozen) watercress was toxic to *G. pseudolimnaeus* (48-hr LC_{50}s: ca. 1 g wet/liter), and the primary volatile secondary chemicals released by damage were highly toxic. The predominant

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glucosinolate hydrolysis product, 2-phenylethyl isothiocyanate had 48-hr LC_{50}s between 0.96 and 3.62 mg/liter, whereas 3-phenylpropionitrile was less toxic, with 48-hr LC_{50}s between 130 and 211 mg/liter. These results suggest that live watercress is chemically defended against consumption. The glucosinolate-myrosinase system, recognized as the principle deterrent system of terrestrial crucifers, is also possessed by *N. officinale* and may contribute to defense from herbivory by aquatic crustaceans. This system may be just one of many examples of the use of defensive chemicals by stream and lake macrophytes.

Key Words—*Nasturtium officinale, Rorippa nasturtium-aquaticum, Gammarus pseudolimnaeus*, freshwater, streams, herbivory, chemical defense, glucosinolates, phenylethyl isothiocyanate.

INTRODUCTION

Invertebrate herbivory on live macroscopic plants is conspicuously rare in freshwater systems (Otto and Svensson, 1981; Wetzel, 1983; for exceptions see Gaevskaya, 1969; and Sheldon 1987). Vascular macrophytes are important as both invertebrate habitats and as substrata for epiphytic periphyton (e.g., Hutchinson, 1975; Gregg and Rose, 1985). When these plants die or senesce, i.e., when tissues rapidly decompose (Webster and Benfield, 1986), they contribute directly to the particulate energy base of aquatic systems, providing energy to detritivores or decomposers (Wetzel, 1983; Mann, 1988).

Despite low herbivory on macrophytes, few have investigated plant–animal interactions from a chemical standpoint, which has resulted in a common opinion that chemical defenses are relatively unimportant in freshwater macrophytes (McClure, 1970; Hutchinson, 1975). Recently, Lamberti and Moore (1984) noted that there is insufficient information available to assess the importance of antiherbivore chemicals in freshwater plants, although Otto and Svensson (1981) and Ostrofsky and Zettler (1986) have argued for a greater role of secondary chemicals in freshwater plant defense. Most recently, Hay and colleagues (see Hay and Fenical, 1988, for a review) have shown that secondary chemicals are very important determinants of seaweed use by marine herbivores. Our study of watercress [*Nasturtium officinale* R. Br. – synonym: *Rorippa nasturtium-aquaticum* (L.) Hayek], a common spring-brook macrophyte, and *Gammarus pseudolimnaeus* Bousfield, a commonly cooccurring amphipod, suggests that this macrophyte is chemically defended from herbivory and that the defensives act in a manner comparable to terrestrial relatives.

Watercress is a crucifer that possesses glucosinolates and myrosinase, which are common to the family (Kjaer, 1976). It was introduced to North America in the 1700s from Europe and is now widely distributed in the United States (Green, 1962) in alkaline springs and spring-fed streams, where it often establishes extensive beds (Howard and Lyon, 1952; Voss, 1985). Because of

its use as a food for humans, much information is available on watercress aroma and taste-related chemistry. Glucosinolate and myrosinase are stored separately in the plant, but upon tissue damage, glucosinolate hydrolysis is mediated by myrosinase to yield characteristic isothiocyanates and nitriles (Van Etten and Tookey, 1979; Larsen, 1981). In watercress, 2-phenylethyl isothiocyanate and 3-phenylpropionitrile are the predominant hydrolysis products (MacLeod and Islam, 1975; Spence and Tucknott, 1983) and constitute 25–55% of the volatile compounds. The 2-phenylethyl isothiocyanate is considered the predominant flavor component and imparts the characteristic "hot" taste of watercress (Freeman and Mossadeghi, 1973).

In terrestrial studies, glucosinolates and corresponding isothiocyanates of crucifers are considered classical examples of chemical defense (Feeny, 1976, 1977). These compounds are deleterious to nonadapted herbivores (e.g., Lichtenstein et al., 1964; Blau et al., 1978) and will reduce herbivore damage (Louda and Rodman, 1983; Louda et al., 1987). Moreover, isothiocyanates are released as a response to direct damage of tissues, underscoring the causal relationship with herbivory. Isothiocyanates are especially noxious and can be quite toxic (Lichtenstein et al., 1964; Lowe et al., 1971; Åhman, 1986). However, we know of no research on the ecological implications of the mustard oil system in aquatic plants such as watercress.

Amphipods are also commonly found in alkaline spring streams and often occur among watercress (Howard and Lyon, 1952; Hynes and Harper, 1972; Gregg and Rose, 1985). In particular, *Gammarus* are generalist consumers in that they eat other animals, terrestrial leaf litter, algae (Marchant and Hynes, 1981; Sutcliffe et al., 1981), and are able to digest cellulose (Bärlocher and Porter, 1986). *Gammarus* also readily consumes decomposing macrophytes (e.g., Newman et al., 1987), including watercress (Newman, unpublished manuscript).

In this study, we report the results of feeding choice tests with watercress leaves, the concentrations of isothiocyanate yielding glucosinolates in watercress, and the toxicity of frozen watercress and glucosinolate degradation products to *Gammarus pseudolimnaeus*. We suggest that the lack of aquatic herbivory on North American watercress is in large part due to a chemical defense system. Watercress may represent one of many emergent macrophytes that have carried defensive systems into the aquatic environment.

METHODS AND MATERIALS

Observations were made in Connecticut (Squabble Brook, East Canaan, Connecticut; 42°1′N, 73°16′W) and Michigan (Carp Creek, 45°33′N, 84°41′W; T36N, R3W, S4, Cheboygan County, Michigan). Both streams were spring

fed, and typically maintain temperatures of 7–13°C. The waters were alkaline with conductivities between 200 and 300 μS/cm and support extensive watercress beds.

In the course of detritivory experiments, we discovered that watercress possesses noxious compounds that are harmful to *Gammarus* and hypothesized that glucosinolate hydrolysis products (2-phenylethyl isothiocyanate and 3-phenylpropionitrile) were the harmful agents, because senescing yellow leaves have less of the characteristic watercress aftertaste. To evaluate this hypothesis, we performed feeding choice tests with fresh and yellow (senescent) watercress leaves to determine the extent that watercress was palatable to *G. pseudolimnaeus*.

Feeding Choice Test. To determine if *Gammarus* would readily consume fresh watercress, we conducted feeding choice tests, comparing yellowed watercress leaves with fresh green leaves. Preliminary field observation had suggested some damage to senescent yellowed leaves. For these experiments, most yellowed leaves were obtained by keeping leaves in the dark at 10°C for about one week, but some yellowed leaves were collected from plants in the streams. Eight amphipods (ca. 5 mg dry wt. each) were placed into choice chambers (modified 100 × 20-mm Petri dishes, divided into four pie-shaped compartments that were open to one another) with 60 ml filtered stream water. The amphipods were allowed to acclimate to the chambers (10°C) without food for 12 or 48 hr, which resulted in two levels of starvation. Leaf disks (ca. 105 mm^2; 25 mg wet) were cut from fresh and yellowed leaves with a cork borer. Disks were randomly assigned to choice chambers, one disk per compartment, in one of four categories: 3 green disks; 2 green + 1 yellow disk; 1 green + 2 yellow (CT); or 1 green + 1 yellow disk (MI); 2 green + 2 yellow disks. Disks were held in compartments by pinning the disks with insect pins to silicon knobs in each compartment. The amphipods were allowed to feed for 24 hr, the disks were removed, and disk area remaining was computed (by planimeter or digitizer). During each experiment, a set of four green and four yellow disks were placed in chambers without amphipods and served as controls to estimate initial areas. For each choice type there were 6–12 replicates at 12- and 48-hr starvation periods.

Damaged Tissue Tests. Toxicity tests were conducted with frozen watercress to determine the presence of harmful chemicals. Mustard oils are typically in minute concentrations prior to plant damage. Freezing, however, produces extensive plant damage and, upon thawing, promotes the hydrolysis of glucosinolates to the corresponding isothiocyanates and nitriles.

Fresh watercress leaves were clipped from the plants, washed, blotted dry, and then frozen at ca. −15°C. Toxicity tests were conducted in 100 × 15-mm plastic Petri dishes containing 40 ml of filtered stream water. Ten amphipods (ca. 5 mg dry wt. each) were placed in each Petri dish and acclimated for 24

hr in an environmental chamber at ambient stream temperature (ca. 10°C). The frozen leaves were thawed and predetermined wet weights of leaf tissue were randomly placed into each dish, which was then covered. Triplicate dishes of each of five concentrations (a control and four levels of watercress) were used in each toxicity assay. Mortality was observed over time for 48 hr. Lethal concentrations (LC_{50} values) and confidence intervals were computed with the trimmed Spearman-Karber method (Hamilton et al., 1977).

Toxicity of Secondary Compounds. Toxicity tests were also conducted with the two secondary compounds known to be prevalent in watercress: 2-phenylethyl isothiocyanate and 3-phenylpropionitrile (Aldrich Chemical Co., Milwaukee, Wisconsin). Experimental conditions were identical to the damaged tissue tests. Because the compounds were volatile oils, serial dilutions were made with absolute ethanol and appropriate concentrations were applied to inert filter disks (Reeve Angel Cellulose disks). Filter disks with the appropriate amount of compound were placed in each Petri disk (concentrations of 0.1–25 mg/liter for 2-phenylethyl isothiocyanate and 25–375 mg/liter for 3-phenylpropionitrile), three replicate dishes per concentration. Filter disks with an amount of ethanol equal to that in the lowest concentration (highest dilution) were placed in the control dishes. The experiments were then conducted and analyzed in the same manner as the frozen watercress experiments.

Chemical Analyses. Watercress was analyzed for carbon, hydrogen, nitrogen, and isothiocyanate-yielding glucosinolates during summer 1988 in Michigan. Fifteen subsamples (2–4 mg, dry) of yellowed and green leaves were dried (65°C) and analyzed for carbon, hydrogen, and nitrogen with a Perkin-Elmer 24000 CHN Total Elemental Analyzer. The subsamples were taken from leaflet material remaining after disks were cut for the choice experiments and represent the range of material used in the choice tests. A sample of watercress roots as also analyzed.

Glucosinolate content was analyzed for frozen leaves as well as fresh green and yellowed leaves used in the choice experiments. Samples of fresh and yellowed leaves (0.8–2.6 g wet) were obtained from leaf material remaining from different choice tests and immediately boiled for 10 min in 70% methanol. Glucosinolate analyses were done (within three months) according to the methods described by Blua (1984) and Blua and Hanscom (1986), except that phenylethyl isothiocyanate was used as the standard for isothiocyanate concentration determination. Results are expressed as milligrams of phenylethyl isothiocyanate-yielding glucosinolate (IYG) per gram (wet) watercress. Concentrations of phenylethyl isothiocyanate from the tissues can be determined by multiplying the IYG concentrations by 0.371. Frozen watercress was analyzed for phenylethyl isothiocyanate directly, without fixation or the standard myrosinase treatment. Subsamples of thawed, frozen watercress (0.8–2.6 g wet) were placed in 40 ml cold water, and 10 ml methylene chloride was then added and the bottles

were shaken vigorously to extract the released isothiocyanates. The methylene chloride phase was later processed (within three months) and analyzed by GC for isothiocyanate content in the same manner as done for the fresh and yellowed samples. Standard extraction procedures and analysis (e.g., Blua and Hanscom 1986) of the thawed leaf tissue remaining after methylene chloride extraction revealed no detectable glucosinolates. A set of standards prepared on filter disks (similar to the toxicity test disks) was treated in the same way as the frozen watercress samples; analysis showed almost 100% recovery.

RESULTS

Watercress Chemistry. Fresh green watercress contained the highest concentrations of carbon, hydrogen, and nitrogen, with total nitrogen >5% and C/N ratio of 7.27 (Table 1). Yellow leaves had significantly lower carbon and nitrogen (2.7%) with a C/N ratio of 12.7. Roots had the lowest nitrogen and the highest C/N ratio (16).

Fresh green watercress also had much higher concentrations of phenylethyl glucosinolate (8.9 mg/g) than yellowed leaves (1.2 mg/g; $P < 0.001$) (Table 2). Frozen green watercress yielded phenylethyl isothiocyanate concentrations nearly identical to fresh watercress ($P > 0.5$), which suggests that freezing and thawing resulted in nearly complete hydrolysis of the phenylethyl glucosinolate to isothiocyanate.

Choice Tests. Amphipods readily consumed yellowed leaf disks (typically 40–60% of the area offered), but rarely consumed more than 10% of the fresh green disks (Figures 1 and 2). For each choice type (e.g., two fresh and two

TABLE 1. CARBON, NITROGEN, AND HYDROGEN CONCENTRATIONS (% dry wt) IN WATERCRESS LEAVES AND ROOTS

	Green leaves	Yellowed leaves	Roots
N^a	15	15	5
Carbon	37.89	30.18	37.34
2 SE	1.04	0.72	0.64
Hydrogen	6.04	5.45	5.85
2 SE	0.13	0.14	0.13
Nitrogen	5.37	2.70	2.39
2 SE	0.49	0.64	0.31
C/N ratio	7.27	12.74	15.85
2 SE	0.68	1.94	1.93

[a] Number of samples.

TABLE 2. PHENYLETHYL GLUCOSINOLATE CONCENTRATIONS (mg/g wet) YIELDED FROM FRESH GREEN, YELLOWED, AND FROZEN GREEN WATERCRESS IN MICHIGAN, 1988[a]

	Green leaves	Yellowed leaves	Frozen green
N	11	12	6
mg/g	8.85	1.24	9.55
2 SE	2.22	0.48	1.38

[a] *N* = number of samples. Phenylethyl isothiocyanate concentrations can be obtained by multiplying by 0.371.

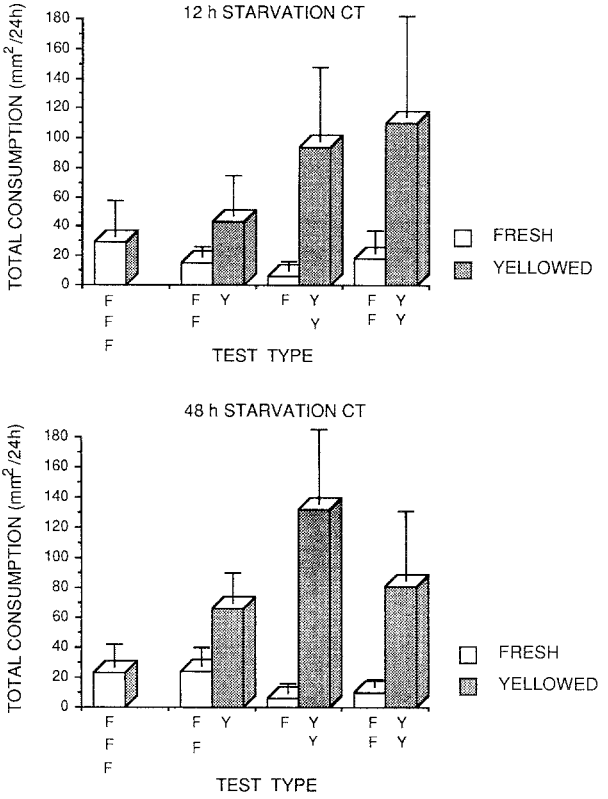

FIG. 1. Consumption (area/24 hr) of fresh green (F) and yellowed (Y) leaf disks by *Gammarus pseudolimnaeus* in Connecticut, November 1987, after 12- and 48-hr starvation periods. Test type indicates the number of fresh and yellow disks offered. Vertical lines represent +2 SEs.

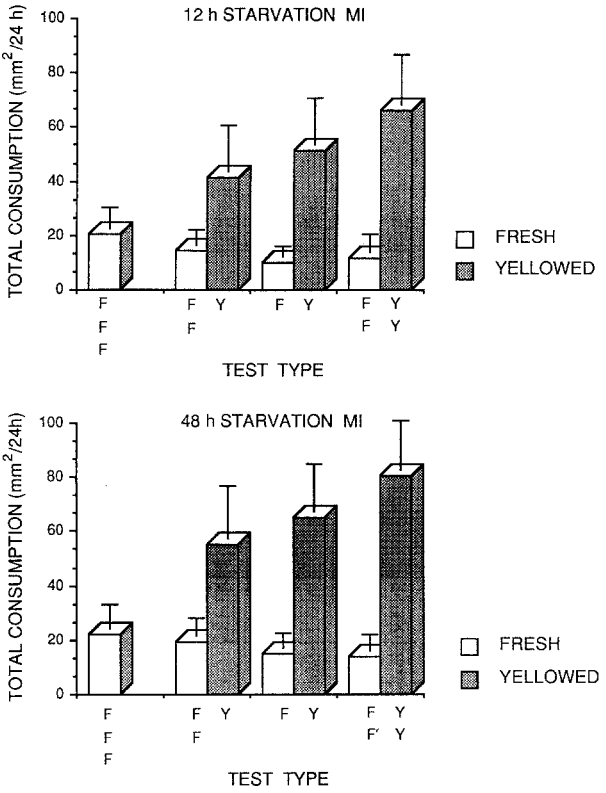

FIG. 2. Consumption (area/24 hr) of fresh green (F) and yellowed (Y) leaf disks by *Gammarus pseudolimnaeus* in Michigan, July–August 1988, after 12- and 48-hr starvation period. Test type indicates the number of fresh and yellow disks offered. Vertical lines represent +2 SEs.

yellow disks), both total consumption and mean consumption (an average disk) of yellowed disks was significantly higher than that of green disks (*t* tests; all $P < 0.01$). Even when *Gammarus* was starved for 48 hr and provided only green disks, total consumption was less than half that of yellow disks. Starvation duration (12 or 48 hr) did not increase the consumption of leaf disks in Connecticut but did increase consumption significantly in Michigan ($P < 0.05$). Consumption of cress (both green and yellow) by amphipods in Michigan and Connecticut was not significantly different ($P > 0.05$).

Damaged Tissue Tests. Frozen watercress was toxic to the amphipods. Amphipod mortality showed typical dose–response curves, with no mortality at the lowest concentrations, partial mortality at intermediate concentrations, and

TABLE 3. TOXICITY OF FROZEN WATERCRESS TO *Gammarus pseudolimnaeus*[a]

	48-hr LC_{50} (mg/liter)	95% CL (mg/liter)
Leaves		
Michigan (August 1987)	475	420–540
Connecticut (November 1987)	1122	996–1262
Michigan (July 1988)	928[b]	871–990
Roots		
Michigan (July 1988)	967	863–1084

[a] Forty-eight-hour LC_{50} values were computed with the trimmed Spearman-Karber method, with four levels (plus controls) per test and three replicates per level. All concentrations are for wet weight.

[b] Combined results of two tests; eight levels plus controls.

complete mortality at the highest concentrations. The 48-hr LC_{50} values for leaves ranged from 475 to over 1000 mg (wet)/liter (Table 3). Toxicity of roots was similar to leaves. Observations during the tests suggested that most mortality occurred within 24 hr; 24-hr LC_{50}s were $<30\%$ larger than 48-hr LC_{50}s. Toxic levels of frozen cress appeared to result in abnormally high rates of gut evacuation, which were not seen at low and control levels. New amphipods introduced into the highest concentrations after the 48-hr experiments were also killed, but the toxic action was reduced.

Toxicity of Secondary Compounds. Previous research demonstrated that 2-phenylethyl isothiocyanate and 3-phenylpropiontrile are predominant glucosinolate hydrolysis products in watercress (MacLeod and Islam, 1975; Cole, 1976; Spence and Tucknott, 1983). Forty-eight-hour LC_{50} values for 2-phenylethyl isothiocyanate were <4 mg/liter (Table 4). 3-Phenylpropionitrile was not as toxic, giving LC_{50} values over 100 mg/liter (Table 4). Toxic effects were similar to frozen watercress; mortality occurred rapidly and high rates of defecation were noted. The 24-hr LC_{50} values were not significantly different from 48-hr LC_{50} values. With 3-phenylpropionitrile, however, amphipods at intermediate concentrations appeared partially paralyzed, but did not die even after 96 hr.

DISCUSSION

Field-collected yellowed watercress typically lacked the bite and odor characteristic of green watercress and was consumed by *G. pseudolimnaeus*, which strongly preferred yellowed watercress to green watercress, despite the

TABLE 4. TOXICITY OF *Nasturtium officinale* SECONDARY COMPOUNDS TO *Gammarus pseudolimnaeus*[a]

	48-hr LC_{50} (mg/liter)	95% CL (mg/liter)
2-Phenylethyl isothiocyanate		
Michigan (August 1987)	3.62	2.77–4.74
Connecticut (November 1987)	0.96	0.67–1.36
Michigan (July, 1988)	1.33	0.97–1.82
3-Phenylpropionitrile		
Michigan (August 1987)	130	N/A[b]
Michigan (July 1988)	211[c]	193–230

[a] Forty-eight-hour LC_{50} values were computed with the trimmed Spearman-Karber method, with four levels (plus controls) per test and three replicates per level.
[b] Confidence intervals not computable.
[c] Combined results of two tests; eight levels plus controls.

higher nitrogen content of the latter, and usually consumed five times more yellowed watercress. Fresh green watercress, however, had phenylethyl glucosinolate concentrations that were seven times higher than yellowed watercress. Cell-damaged green watercress was toxic to *G. pseudolimnaeus*. The primary volatile secondary chemicals released by damaged watercress were also highly toxic to *G. pseudolimnaeus*. These results, although only correlative, suggest that live watercress is chemically defended against consumption and that the glucosinolate–myrosinase system (recognized as the principle deterrent system of terrestrial crucifers) is also active against aquatic crustacean herbivores. Further work is needed to determine if other possible explanations that we did not test for, such as mechanical defense or other chemicals, can be eliminated.

Gammarus and watercress both occur in spring-fed alkaline streams. *G. pseudolimnaeus* is often most abundant in the watercress beds, living among the roots and submerged leaves (Hynes and Harper, 1972; Newman, personal observation). These crustaceans are generalist feeders, consuming algae, other animals, and terrestrial leaf litter (Sutcliffe et al., 1981). Moreover, *Gammarus* reportedly possesses the ability to digest cellulose (Bärlocher and Porter, 1986). Therefore, *G. pseudolimnaeus* has the potential to consume watercress, yet little plant damage was observed on green leaves, only on senescing yellow leaves. Others have reported occasional feeding. For example, Minckley (1963) reported that *G. minus* and *G. bousfieldi* contained watercress in their guts, but did not identify whether the material was from living tissue or senescent leaves.

H.B.N. Hynes (personal communication, University of Waterloo) notes that in England, after commercial watercress beds are cleared for replanting, *G. pulex* (indigenous to Europe) will consume newly planted watercress cuttings; however, all other food sources are removed during clearing. Sutcliffe et al. (1981) found poor to moderate growth of *G. pulex* on watercress in laboratory trials but also noted high (up to 95%) mortality. The only reported aquatic pest of watercress known to us is the trichopteran *Limnephilus lunatus*, which is indigenous to Europe (Gower, 1967).

As mentioned earlier, in our work, the yellowed cress usually lacked the bite and odor characteristic of fresh watercress and had greatly reduced levels of glucosinolates. Frozen watercress leached in the stream for two days was not toxic to the amphipods and was consumed. Likewise, Feeny and Rosenberry (1982) noted that yellowed senescent leaves of the terrestrial crucifer *Dentaria* sp. had no detectable glucosinolates. Loss of toxic volatile compounds conicided with marked increase in consumption. *G. pseudolimnaeus* strongly preferred yellow leaves to green leaves, typically consuming over 50% of a yellowed leaf disk, but rarely more than 15% of a green disk. The yellowed disks often lost large areas, whereas *G. pseudolimnaeus* only sampled the edges of green disks, even when no other food was available. The average amount of consumption of individual green leaf disks was similar among test types, further suggesting a sampling of each disk rather than sustained consumption.

Food quality does not appear to explain the lack of consumption of the green leaves. Most aquatic invertebrates, including *Gammarus*, prefer food with high nitrogen and low C/N ratios (Bird and Kaushik, 1981). Fresh leaves had a high nitrogen content (higher than most terrestrial and aquatic plants reported by Otto and Svensson, 1981) and a low C/N ratio (yet similar to the ratio of 7.5:1 reported for watercress by Dawson, 1980), which suggest a high food quality. In contrast, yellowed cress had significantly lower nitrogen and higher C/N ratios. Therefore, choice seems unrelated to food quality.

In our experiments, cell damage via freezing leached substances toxic to *G. pseudolimnaeus*. Freezing damaged the plant tissue and resulted in high production of glucosinolate hydrolysis products (phenylethyl isothiocyanate) upon thawing. Freezing and thawing apparently resulted in complete hydrolysis of the glucosinolate. The primary products of glucosinolate hydrolysis in watercress are 2-phenylethyl isothiocyanate and the corresponding nitrile (3-phenylpropionitrile; Freeman and Mossadeghi, 1973; MacLeod and Islam, 1975; Spence and Tucknott, 1983). Cole (1976) determined that, after autolysis, watercress produced 74 μg of 2-phenylethyl isothiocyanate per gram of watercress and reported no other isothiocyanates or nitriles. Our results showed much higher levels of phenylethyl isothiocyanate, about 3.3 mg/g. Others have reported additional volatiles, but in all cases, 2-phenylethyl isothiocyanate was the predominant compound (Freeman and Mossadeghi, 1972, 1973; Spence and

Tucknott, 1983). Moreover, Freeman and Mossadeghi (1972) confirmed that 2-phenylethyl isothiocyanate was the chemical responsible for the characteristic odor and "hot" taste of watercress. By comparing our toxicity results to plant phenylethyl isothiocyanate concentrations, it can be seen that the toxicity of frozen cress corresponds well (within a factor of 3) with the toxicity and amount of phenylethyl isothiocyanate that would be released upon thawing (Table 2).

The isothiocyanates have been proposed to be the primary defensive agent in terrestrial crucifers (e.g., Feeny, 1977; Louda and Rodman, 1983). Studies with terrestrial invertebrates, mostly insects, have shown 2-phenylethyl isothiocyanate to be quite toxic (e.g., Lichtenstein et al., 1964; Åhman, 1986); Lowe et al. (1971) reported the toxicity to dipteran larvae (*Inopus rubriceps*) to be at the level of DDT toxicity (a highly toxic insecticide). Our toxicity tests with 2-phenylethyl isothiocyanate and 3-phenylpropionitrile confirm that both are quite toxic to crustaceans. The 48-hr LC_{50} for 2-phenylethyl isothiocyanate (ca. 1 mg/liter) is comparable to the level of toxicity of moderately toxic insecticides to *G. pseudolimnaeus* (Mayer and Ellersieck, 1986). Toxicity, feeding, and chemical analyses suggest that *G. pseudolimnaeus* food choice may be influenced by the presence of defensive chemicals such as 2-phenylethyl isothiocyanate that are released from watercress upon tissue damage. This would appear to be an aquatic analog to the terrestrial crucifer defense system often cited as a classic example of plant defense. We do not know the toxicity of consumed watercress, however. Neither can we rule out mechanical or other chemical differences that may be correlated with senescence and loss of glucosinolates. Thus, more work is needed to determine more positively if the glucosinolate system is the defensive agent.

The subject of chemical defenses in freshwater systems is largely unexplored. A few incomplete studies suggest that chemical defenses may be highly important. For example, LaLonde et al. (1979) showed that the freshwater alga *Cladophora* possesses fatty acids toxic to dipteran larvae but did not look at food choice or consumption. Similarly, Ostrofsky and Zettler (1986) showed that many aquatic macrophytes possess significant concentrations of alkaloids, but did not relate chemistry to defense. Finally, Otto and Svensson (1981) suggested that the lack of herbivory on aquatic macrophytes was due to defensive chemicals, but they did not relate chemistry to feeding observations. We believe that chemical defenses may be more important in aquatic plants than previously thought and suggest that watercress is only one example of many chemically defended freshwater plants.

This suggestion has important implications for detritivory. Stream invertebrates are quite adept at using detrital material of both autochthonous and allochthonous origin. Aquatic macrophytes decay quite rapidly compared to terrestrial leaf litter (Bird and Kaushik, 1981; Webster and Benfield, 1986) and can be an important component of aquatic energy budgets (Wetzel, 1983; Car-

penter and Lodge, 1986). One possible reason that macrophytes are not consumed until senescence or death is that they are chemically defended (Smock and Stoneburner, 1980). Watercress has a very rapid decay rate (Bird and Kaushik, 1981; Webster and Benfield, 1986), which is in large part influenced by abundance of shredders such as *G. pseudolimnaeus* (Newman, unpublished manuscript). Our results suggest that the reason watercress contributes directly to stream invertebrate energetics only upon senescence and death is that this potentially nutritious food source is chemically defended while alive. The loss of noxious compounds after senescence permits rapid decay and high consumption by stream invertebrates.

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PEPTIDE-MEDIATED BEHAVIORS IN MARINE ORGANISMS
Evidence for a Common Theme

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Abstract—Biology has many common themes such as DNA, RNA, the other biopolymers, and their building blocks. Chemical communication systems have similar common themes. An example is the common usage of amino acids, sugars, and nucleotides as food cues. It is likely that communication systems began with the evolution of specific meanings for preexisting molecules. One class of molecules used in specific communication is peptides. These peptides are generated as part of the body odor of organisms in aquatic environments and can act at a distance or by contact. Evidence is given for a common type of peptide receptor system. Four peptide-mediated behaviors, three in diverse crustacean groups and one in a mollusc, are discussed. The behaviors are of major survival importance, are cued by nanomolar concentrations of peptides generated by serine proteases, and the basic carboxy terminal amino acid is required. The behaviors include attraction to new shells, attraction to living prey, release of larvae, and induction of larval settlement behavior. Studies with pure di- and tripeptides show the same molecules evoke larval release behavior, shell attraction responses, and larval settlement behavior. The pure compounds are effective at nanomolar or lower concentrations. Similar peptides function as specific cues in vertebrates in response to wounding. Thus, peptide communication using serine protease-generated peptides appears to be a common theme.

Key Words—Trypsin, behavior, crustaceans, pheromones, molluscs, predator–prey interactions, peptides, serine proteases.

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INTRODUCTION

Aquatic chemical ecology excels in studies using polar comopunds. Recent technological advances in the synthesis, sequencing, and analysis of peptides facilitate studies and use of these polar compounds in studies of novel roles of peptides. This area should be a very productive one in the next decade.

The extensive regulatory roles of peptides in human metabolism are one aspect of their bioactivity. Other aspects are less well understood. The mediation of behavior by peptides is a new frontier. This phenomenon has been clearly demonstrated in isolated behaviors of aquatic crustaceans and molluscs. That similar mediation of events is observed in the defensive events that occur in response to wounding of vertebrates suggests that there may be classes of peptide receptors that are comparable to those analogous to amino acid receptors. However, the peptide receptors function to cue behaviors other than feeding. It should be emphasized that the examples presented are marine, but that any interaction that enables communication by polar compounds (licking or touching or eating, for example) provides the potential for peptide mediation of behavior. Examples are not yet available from other than the systems discussed today.

Pivotal events in the lives of organisms are not left to chance. Frequently, critical events in an organism's life are associated with stereotyped or precisely timed behaviors. Chemical signals coordinate many such stereotyped behaviors and precisely timed events. For terrestrial organisms, many of the coordination chemicals are volatile compounds (Wilson, 1970). In contrast, most of the chemicals known to coordinate behavior in aquatic organisms are polar compounds, and many of these are peptides (Rittschof and Bonaventura, 1986).

Peptides coordinate a potpourri of behaviors for aquatic organisms. These include gregarious settlement and metamorphosis of many marine invertebrate larvae (Crisp, 1984; Burke, 1984; Morse, 1984; Rittschof, 1985); precisely timed larval release by crustaceans (Forward et al., 1987); location of the population limiting resource (empty gastropod shells) for anomuran crustaceans (McClean, 1974); alarm responses in gastropods (Snyder, 1967; Atema and Burd, 1976); and location of living, intact prey by predatory snails (Rittschof et al., 1984).

The biological events listed above are highly specific and stereotyped responses mediated by peptides, but other similarities are not obvious at the organismal level. Some of the behaviors, however, are related by the mechanism responsible for their coordination. The gregarious settlement of barnacles, larval release behavior in mud crabs, hermit crab shell attraction, and creeping responses of predatory snails have striking similarities in the molecular mechanisms that coordinate them. Those behaviors are all mediated by peptides that can be generated by trypsinlike serine proteases. It appears that this provides a

common theme: the use of peptides, generated by degradative processes, that contain a number of neutral residues and a basic residue (arginine or lysine) at the carboxy terminus.

This theme of a common mediation system will be documented by the presentation of five biological examples. Four are the marine invertebrate examples mentioned above. The fifth is the mammalian complement system and the functions of C3a–C5a peptides. The mammalian example is included because it is well understood and extends the notion of the common theme to the vertebrates. Because each example is dependent upon a different biological system, the information communicated is slightly different in each case. All of the individual stories are fragmentary; however, the aggregate information suggests that there is a common theme in the generation and detection of these peptide signal molecules. One should not lose sight of the fact that the examples are a selected subset and that a variety of equally important behaviors do not work by the proposed mechanism.

The biological examples supporting the theme are diverse. In the short sections that follow, thumbnail sketches of each system are included as a frame of reference. Emphasized in each sketch is the benefit of the biological response to the organism.

SHELL LOCATION BY ANOMURAN CRUSTACEANS

In marine environments, death by predation is the rule rather than the exception. Events such as the death of a snail have added significance because the vacant shell is cycled and recycled through communities as habitats for fish, octopuses, fouling organisms, and crabs (McClean, 1974). Shells are the population-limiting resource for the first marine example, hermit crabs (Vance, 1972).

Hermit crabs use shells for protection. The fit of the shell can influence growth (Frotheringham, 1976) and reproduction (Childress, 1972; Bach et al., 1976). Searching for and acquiring new and better-fitting shells dominates the life of juvenile and adult crabs. In addition to complex behaviors related to assessing and trading shells (Hazlett, 1966, 1981), hermit crabs can chemically locate the source of a new shell from a distance. Molecules released by the flesh of the snail attract hermit crabs, generally those whose shells are not adequate (Rittschof, 1980a), and those belonging to species that usually occupy that type of shell (Rittschof, 1980b; Gilchrist and Abele, 1984). The crabs do not feed on the snail flesh. Rather, they interact with other attracted crabs, take a position in a size hierarchy of the crabs, and wait for the new shell to be released by the predator (McClean, 1974, 1975). When the new shell is released, all crabs in shells smaller than the new shell switch to the next larger shell. This ritual is

repeated whenever a snail loses its life and is a major route of influx of new shells into the population (Wilber and Herrnkind, 1982, 1984).

PREY LOCATION BY GASTROPOD *Urosalpinx cinerea*

Unlike terrestrial predators, many marine predators are slow moving and do not depend upon vision for prey location. Oyster drills, *Urosalpinx cinerea* are slow-moving, virtually blind predatory snails (Frederighi, 1931). The prey of *U. cinerea* are sessile and gregarious. Where prey occur, they are often dense and occur in continuous sheets or reefs. Predators such as oyster drills prey upon these sessile prey much as ungulates graze upon grass. These snails locate living intact prey from a distance by their odors (Carriker, 1955). The type of prey consumed determines the reproductive output of the snail (Frederighi, 1931). The crawl-away juvenile oyster drill is attracted only to barnacles (Rittschof et al., 1985). Survival of juvenile snails is strongly dependent upon locating prey.

AGGREGATIONS OF THE BARNACLE *Balanus amphitrite*

Acorn barnacle adults, *Balanus amphitrite*, are simultaneous hermaphrodites that do not generally self-fertilize (Holm, personal observation). Gregarious settlement in barnacles increases the probability that an individual will reproduce. Settlement is mediated via chemical cues. Larvae respond to chemical cues in two ways: (1) they alter their behavior; or (2) they attach permanently to a surface to which the chemical cue is adsorbed. The biochemical events of metamorphosis are then initiated (Crisp and Meadows, 1962, 1963; Rittschof, 1985).

LARVAL RELEASE BY BRACHYURAN CRABS

Mud crabs, *Rhithropanopeus harrisii*, are the best understood of any brachyuran crustacean with respect to larval release behavior (Forward, 1987). Female crabs extrude and glue fertilized eggs to their abdomens. Embryos are carried until the time of hatching. Hatching is synchronous and precisely timed with respect to lunar, diurnal, and tidal cycles. Females perform a stereotyped larval release behavior in response to pheromones originating from hatching eggs (Forward and Lohmann, 1983; Rittschof et al., 1985; Forward et al., 1987). The synchrony of larval release is thought to reduce the risk of predation and to ensure that the physiologically delicate larvae are introduced into environmental conditions that maximize the probability of successful development.

MAMMALIAN COMPLEMENT CASCADE

In mammals, wounding or infection result in a cascade of biochemical responses. Peptides resulting from proteolytic activation of the complement cascade have a variety of functions (for review, Schiffman, 1982). These peptides induce anaphylaxis, smooth muscle contraction (C3a; Unson et al., 1984), and attract neutrophils (C5a; Chenoweth and Hugli, 1978). These responses, as well as several others, are central to defense against microbial invasion (Schiffmann, 1982).

SYNTHESIS

The stereotyped responses in each example described above have been used as bioassays. Table 1 summarizes the measures used in each bioassay and provides references for the details of each assay. These bioassays have been used to direct the purification of the chemical signals, and to ask questions about the nature, origin, and specificity of the cues mediating responses.

The biological responses to chemical stimulation in the context of Table 1 are all extremely simple. Although the measures examine such basic characteristics as movement, they are extremely sensitive and effective because each response is highly specific. For example, studies of simulated gastropod predation sites show that hermit crabs respond only to flesh from gastropods and that the species of hermit crab attracted is dependent upon the species of snail used to generate the peptide cues (McClean, 1975; Rittschof, 1980a,b; Hazlett and Hernnkind, 1980). Similarly, oyster drills are stimulated to creep only by the odor of balanoid barnacles (Rittschof, 1985). This snail has been placed in the outflow of a biochemical separation column and used as a detector because

TABLE 1. ASSAY MEASURES IN FIVE BIOLOGICAL SYSTEMS

Organism	Assay	Reference
Barnacles	Larval chromatography Settlement	Rittschof et al., 1984
Crabs	Pumping behavior	Forward and Lohmann, 1983
Hermit crab	Attraction	Rittschof, 1980a
Vertebrate wounding	White cell movement Skin swelling Ileum muscle contraction	Schiffmann, 1982 Hugli and Erickson, 1977 Unson et al., 1984
Snails	Directed movement in flow	Rittschof et al., 1983

it will sit for hours in flow without moving until stimulated chemically to creep (Rittschof et al., 1984). Similar extreme specificity is observed in the responses of barnacles (Larman et al., 1982), ovigerous crabs (Forward and Lohmann, 1983; Forward et al., 1987), and the responses to C5a and C3a peptides (Schiffmann, 1982).

In the four marine examples, the behavioral response to chemical stimuli generally requires additional sensory input (Table 2). The best studied example of this is the response of *Urosalpinx cinerea* (Brown and Rittschof, 1984). In this case, either chemical cues alone or flow alone do not evoke creeping. Rather, snails creep only in response to certain levels of flow and certain levels of chemical stimulus. At flows above a certain level or amounts of chemical above a certain concentration, the creeping response is inhibited.

In many instances the precise protein source of the peptide cue has not been identified. It is clear, however, that these cues are generated by trypsinlike serine proteases. In the vertebrate examples the enzymes generating the peptides are known. Digestion of protein sources with trypsin generates biologically active peptides that attract hermit crabs (Rittschof, 1980b), induce metamorphosis in barnacles (unpublished data), and induce hatching and larval release behavior in ovigerous crabs (Rittschof and Forward, 1988). Similar enzymes are responsible for the generation of biologically active peptides in the mammalian complement system (Schiffmann, 1982).

Additional evidence supports the conclusion that proteolytic clips are gen-

TABLE 2. COMPARISON OF RESPONSES, CUE TYPE, AND CUE GENERATION IN FIVE EXAMPLES[a]

	System				
Topic	Hermit crab	Predatory snail	Barnacle larvae	Ovigerous crab	Vertebrate wound
Response	attraction shell switch	attraction	sticking settlement metamorphosis	pumping	attraction contraction anaphylaxis
Cues	peptides + flow	peptides + flow	peptides + surface + flow	peptides + eggs	peptides prostaglandins
Cue generation	serine protease (digestion)	?	serine protease (molting?) (bacteria?)	serine protease (hatching)	serine proteases (wounding)

[a]References in which the original work was presented are given in the text.

erated by trypsinlike enzymes. Carboxypeptidases that degrade arginine or lysine carboxy termini rapidly destroy biological activity in the snail creeping assay (Rittschof et al., 1984), the barnacle settlement assay (Tegtmeyer and Rittschof, 1988), and the crab pumping assay (Rittschof et al., 1985). Removal of the carboxy terminus greatly reduces the effectiveness of the leukocyte attractant peptide (Schiffmann, 1982). Carboxy terminal arginine is required for ileum contraction and the anaphylaxis response associated with the C3a peptide (Unson et al., 1984) (Table 3).

The concentration threshold of leukocyte attractant peptides is in the picomolar range (Chenoweth and Hugli, 1978). Because analysis of the other biologically active peptides is not complete, only conservative estimates can be made for the potency of the native peptides in the marine examples. The threshold concentration for the peptides in the marine examples is in the nanomolar range in all instances where sufficient information is available to make the determination (Rittschof et al., 1983, 1985; Rittschof, 1985).

In a similar fashion, molecular size estimates of the native compounds are imprecise due to limitations of the bioassays and the ongoing nature of the studies. However, there is no doubt that barnacle and snail attractants are 3000- to 5000-dalton peptides, while the crab larval release pheromones are <500 daltons. Molecular size may be related to the biochemical complexity of the environment and the effective distance over which the cue must transmit information. Although all the peptides are relatively stable chemically, they have very short half lives in the marine environment due to biological consumption (Rittschof et al., 1983; Forward and Lohmann, 1983).

TABLE 3. COMPARISON OF PEPTIDE CUE SIZE, IMPORTANCE OF CARBOXYL TERMINAL RESIDUE, TYPE OF CARBOXYL TERMINAL RESIDUE, AND RELATIVE POTENCY IN FIVE EXAMPLES[a]

	System				
Topic	Hermit crab	Predatory snail	Barnacle larvae	Ovigerous crab	Vertebrate wound
Peptide Size	<500 D	<5,000 >1,000	<5,000 >1,000	<500	>7,700
C terminal important	?	yes	yes	yes	yes
C terminal	Arg Lys	Arg Lys	Arg Lys His	Arg Lys	Arg Lys
Potency	?	≤nM	≤nM	≤nM	pM

[a] References for the original work are given in the text.

In addition to work with native peptides, synthetic peptides have been used to probe the responsiveness of barnacle larvae (Tegtmeyer and Rittschof, 1988), ovigerous crabs (Forward et al., 1987; Rittschof et al., 1988), and the vertebrate system (Schiffmann, 1982; Unson et al., 1984) (Table 4). The vertebrate systems have the most stringent requirements for large peptides. The most effective of these peptides, however, evoke responses in the bioassays at the level of the native compounds (Unson et al., 1984).

Of the restricted number of synthetic di- and tripeptides tested for induction of metamorphosis of barnacles, peptides of the general form of neutral–basic or basic–basic are effective at subnanomolar concentrations (Tegtmeyer and Rittschof, 1988).

In bioassays of larval release from ovigerous crabs, neutral–basic and neutral–neutral–basic peptides have effectiveness that is highly composition-dependent (Forward et al., 1987; Rittschof et al., 1988). Peptides possessing arginine at the carboxy terminus are more effective than carboxy terminal lysine peptides. Carboxy terminal arginine tripeptides containing isoleucine and glycine are most potent. Response thresholds to pure peptides span nine orders of magnitude, from femtomolar for the most effective compound (isoleucyl-glycyl-arginine) to submicromolar for the carboxy terminal lysine compounds (Rittschof et al., 1988).

Several biological assays have been used to probe responses to mixtures of amino acids. Micromolar to millimolar binary mixtures of free amino acids elicit biological responses in the ovigerous crab and hermit crab system. In the case of the ovigerous crab, only binary mixtures containing a neutral and a basic

TABLE 4. COMPARISON OF EFFECTIVENESS OF SYNTHETIC PEPTIDES IN EVOKING RESPONSES IN BIOLOGICAL ASSAYS[a]

	System		
Topic	Barnacle larvae	Ovigerous crab	Vertebrate wound
Synthetic peptides	yes	yes	yes
No. of residues[b]	2+	2+	more than 5
Form[c]	N-B B-B	N-B	X-X-N-N-B
Best	?	Ile-Gly-Arg	X-X-Leu-Gly-Arg X-X-Leu-Gly-Arg
Potency	nM	fM	pM

[a] References citing the original work from which the data in this table are given in the text.
[b] Number of amino acids in the peptide.
[c] N = neutral amino acid; B = basic amino acid.

amino acid are effective (Forward et al., 1987; Rittschof et al., 1988). In the hermit crab system, tests conducted on two species of hermit crab demonstrated that one species responds only to neutral–basic binary mixtures similar to the ovigerous crabs. For this species, the most attractive mixtures contain isoleucine or leucine and lysine. The other species, however, responds to binary mixtures containing leucine and either arginine or lysine. In the latter case, high concentrations of a single amino acid, leucine, are also attractive.

Thus, cleavage products generated by the action of trypsinlike enzymes upon structural proteins (flesh, egg membranes, complement proteins, and possibly upon the exocuticle or cement of barnacles) coordinate highly specific responses. The evolution of cues and pheromones generated from hydrolysis of structural components by one of the most common classes of hydrolytic enzymes, the serine proteases (Desnulle, 1960), is mechanistically simple. The raw materials—enzymes and cleavage products—are integral to life itself. What is required is selection pressure for the ability to detect and respond to the peptide cue. Events that are critical to survival and that are closely associated with cue production are subject to extreme selection pressure. All five of the examples discussed have exactly these characteristics.

It could be argued that the common theme of these chemical signals could lead to confusion of responses or cross-talk between organisms. In fact, both the predatory snail and the barnacle appear to use the same cue for different purposes as both animals respond to the same highly purified material (Rittschof, 1985). In contrast to this long-distance cue, the mud crab pheromone is essentially a very personal pheromone. It is effective at very low concentrations and over very short distances because of rapid dilution due to mixing, and because it is released as a pulse. These features, coupled with the fact that eggs cannot be made to hatch prematurely by the pheromone, ensures that this system is virtually fail-safe. Confusion may also be avoided by spatial separation of sensitive organisms or by high levels of receptor discrimination. In certain instances there is a strong possibility that the simultaneous utilization of a cue by several species may occur. For example, one possibility currently under investigation is that compounds released from snail flesh simultaneously cue both hermit crab attraction and settlement of fouling organisms on the newly available shell.

If all the signal receptor systems have evolved from a common pathway, then other aspects of the best studied systems may also apply to the less well studied systems. For example, leukocytes contain receptors for prostaglandin-like compounds (Schiffman, 1982), and prostaglandin-like substances have also been implicated in crustacean larval release (Clare et al., 1982). This may be more than a coincidence. Studies are currently being conducted to determine if these types of parallels exist.

Indirect actions of enzymes (the generation of biologically active peptides)

are well accepted events in endocrinology. This is exemplified by the brain–gut peptides and the plethora of bioactive hormones possessing a carboxy terminal arginine. The concept that internal communication systems have evolved from external systems is gaining credibility in marine systems (Carr et al., 1987). Demonstration that the biochemistry of some external communication systems is identical to that of the internal systems in the evolutionarily more advanced forms would be strong support for this hypothesis.

In summary, one theme in peptide communication between marine organisms appears to be the use of peptides generated by serine proteases to cue highly specific behaviors. Similar events appear to occur in internal regulation in vertebrates. The hypotheses drawn in this study suggest that similar biochemical processes may also use degradative enzymes to produce chemicals with high information potential for use in internal and external communication.

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LACK OF PREDICTABILITY IN TERPENOID FUNCTION
Multiple Roles and Integration with Related Adaptations in Soft Corals[1]

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Abstract—Terpenoids are commonly found in alcyonacean soft corals of the Great Barrier Reef (Coelenterata, Octocorallia), but they are highly variable in their distribution within this order. Such secondary metabolites in these organisms vary widely in type, concentration, and function. Thus far, they have been found to play roles in predator defense, competitor defense, antifouling, and reproduction. The effectiveness of compounds derived from individual species also varies widely with respect to interactions with other members of the community. These chemical characters function in concert with other morphological, physiological, behavioral, or developmental adaptations that have similar functions. Integration of these adaptations is highly species-specific. The multiple functions of terpenoid compounds and the high degree of species specificity in their ecological roles greatly reduces the ability to predict either the presence or absence of a terpenoid from other related characters. In addition, simple indicators of terpenoid function appear not to exist. If such indicators do exist, they are more likely to be found at higher levels of taxonomic resolution (within families or genera).

Key Words—Terpenoids, soft coral, Alcyonacea, Coelenterata, Octocorallia, defense, predator–prey interaction.

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INTRODUCTION

Secondary metabolites have long been known to play an important role in the ecology of plants and animals (Sondheimer and Simeone, 1970; Rice, 1984). Early accounts of secondary compounds and their functions in nature were drawn from terrestrial ecological studies examining antiherbivore interactions (e.g., Janzen, 1969, 1970, 1971; Harper, 1976; Rosenthal and Janzen, 1979; Rhoades, 1985). In early studies, an antipredator role was often assumed for secondary compounds. Later, other functions were considered, including anticompetitor adaptations such as allelopathy (Muller, 1966) that reduced germination rate in neighboring plants, mate attractants (i.e., pheromones in insects and higher animals; Riddiford and Williams, 1967; Bronson, 1971), trail markers (e.g., in ants; Wilson, 1965), and insect attractants (e.g., Edgar and Culvenor, 1975).

When natural products chemistry was extended to the marine environment (see Scheuer, 1973, 1978a, b, 1980, 1981, 1983), secondary metabolites again were initially assumed to be associated with predator defense and antibiosis/antifouling. Chemical communication, however, also was believed by some to be important in the sea, helping to structure marine and aquatic planktonic communities (Kittredge, 1974; Lucus, 1947, 1949, 1955; Saunders, 1957). Later studies lent support to this hypothesis (e.g., Jackson and Buss, 1975; Strickler, 1982, 1984; Kerfoot, 1982; ses Bakus et al., 1986, for review).

In early chemical ecological studies, it was common that a single role was identified for a given secondary metabolite or set of metabolites. In some cases, the roles of compounds were related to morphological structure or behavior (e.g., Bakus, 1976). Janzen (1979), however, has pointed out that such an approach may be too simplistic.

Alcyonacean soft corals (Coelenterata, Octocorallia) are known to be rich in secondary compounds, especially terpenes (Faulkner, 1977, 1984, 1986, 1987), and this richness is now understood to contribute to their ecological and evolutionary success (Fishelson, 1970; Bakus, 1981; Dinesen, 1983; Coll and Sammarco, 1986; Sammarco and Coll, 1988). These secondary metabolites have a multitude of functions, and enough is now known about the chemical ecology of the Alcyonacea to consider how their secondary metabolites integrate with other adaptations that perform a similar function (Sammarco, 1986, 1987).

In this analytical review, we show how terpenoids in the Alcyonacea and their multiple ecological functions integrate with functionally related adaptations (physiological, morphological, behavioral, etc.) in a highly complex manner. We also demonstrate how this high interspecific variability in chemical function coupled with a similar high variance in related adaptations greatly reduces the ability to predict a priori the ecological role of a secondary compound.

ICHTHYOTOXICITY IN ALCYONACEAN SOFT CORALS

Chemical studies have revealed a richness and diversity of terpenoids in Great Barrier Reef soft corals (Coll et al., 1980, 1985, 1986). Ichthyotoxicity bioassays using the fish *Gambusia affinis* (Yamanouchi, 1955) performed on ~400 specimens from the northern, central, and southern regions of the Great Barrier Reef revealed a consistent frequency of 50% ichthyotoxicity for common, exposed soft corals (Coll et al., 1982b; Coll and Sammarco, 1983, and unpublished data). In most instances, ichthyotoxicity was associated with the presence of terpenoid compounds. (Such tests on "evolutionarily naive" fish, of course, are used simply to detect the presence or absence of toxins. More refined tests performed on ecologically relevant species are used to answer detailed questions about roles of the compounds in their own habitat; see Alino et al., 1988).

Surveys of Great Barrier Reef soft corals have also revealed high interspecific and intergeneric variance in ichthyotoxicity. Within some genera (e.g., *Lemnalia* and *Sarcophyton*), toxicity levels of extracts were consistently high, while those derived from species within other genera (e.g., *Cladiella* and *Anthelia*) were generally nontoxic. Extracts from species within another set of genera (e.g., *Lobophyton* and *Sinularia*) spanned the entire range of toxicity. In addition, there appears to be no obvious relationship between the occurrence of toxicity and characteristics of the environment, biological or physical, from which they were sampled. In short, variance in the frequency of occurrence of ichthyotoxicity in the group is high, and simple indicators of its presence are absent.

ICHTHYOTOXICITY AND ANTIPREDATOR ADAPTATIONS

Relationships between Ichthyotoxicity and Feeding Deterrence. Numerous examples of chemical predator defense may be found in both the terrestrial (Whittaker and Feeny, 1971; Feeny, 1975) and marine (Halstead, 1978) literature. Recent field surveys indicate that soft corals of the Great Barrier Reef are rarely preyed upon by generalist predators, including many fish and crustaceans (Sammarco, unpublished data; P. Alino, work in progress). Yet in an experiment assessing 36 soft corals, we found no significant correlation between ichthyotoxicity (as defined above) and feeding deterrence (La Barre et al., 1986). Toxic characteristics were not correlated with olfactory or palatability responses, nor were these two types of responses correlated with each other. In support of this, recent laboratory feeding experiments have demonstrated that *Chaetodon melanotus* (Pisces), a reef fish specialized to feed on polyps of soft and scler-

actinian corals, responds to visual cues from soft corals, not chemical ones (Alino et al., 1988).

If the secondary metabolites of soft corals are playing a role in taste or olfaction (also see Jones, 1962; Schwartz et al., 1980; Faulkner and Ghiselin, 1983; Rowell et al., 1983), their specific effects could have been masked or augmented by other molecules, such as feeding stimulants, steroidal glycosides (Lucas et al., 1979), or prostaglandins (Gerhart, 1984). Soft corals possess a wide range of primary and secondary metabolites, including those mentioned above (see Pawlik et al., 1987), the effects of which have not been isolated in this context from those of the terpenes. Herein lies fertile ground for future experimentation.

Thus, the presence of ichthyotoxicity in a soft coral is a poor indicator of feeding deterrence.

Relationship between Ichthyotoxicity and Morphological Antipredator Adaptations. Feeding deterrence can also be manifested through morphological adaptations. The Alcyonacea exhibit a wide range of morphological variability (Bayer, 1956). Colonies vary in form from flat and encrusting to erect and branching, and their tissues may be hard and impenetrable, soft and fleshy, or spikey, with the polyps completely protected from predators. If the terpenoids discussed above were functioning as antipredator defenses, one might intuitively expect to find a negative correlation between their presence and the occurrence of physical defense against predators (Bakus, 1974, 1976; Bakus and Thun, 1979).

Soft corals from 19 genera spanning numerous families were assessed for ichthyotoxicity and 16 morphological characters assumed to possess antipredator functions. No negative correlation was found between these two sets of characters. Morphological characters considered included gross colony form, colony texture, presence of mucus, colony color, polyp retractility, and sclerite morphology and distribution. Pattern-seeking analyses also revealed no significant negative associations. In fact, two positive associations emerged, possibly implying multiple (or synergistic) predator defenses.

Reduction of the data set to 56 species drawn only from the genus *Sinularia* and the family Nephtheidae (both of which exhibit a wide range of toxinological and morphological variation) yielded more insightful results. Here, only five characters that were clearly potentially related to predator defense were considered: polyp armament, mineralization of the polypary (upper polyp-bearing portion of the colony), mineralization of the coenenchyme (colony interior), tissue consistency and polyp retractility. The first three were found to be significantly negatively correlated with the presence of ichthyotoxicity. Separate analyses of data from the two sets of taxa further clarified these relationships. Association between toxicity and the first two characters occurred only in the Nephtheidae;

the negative association involving mineralization of the coenenchyme was attributable solely to the genus *Sinularia*.

Thus, the presence of morphological antipredator characters can act as an indicator of the absence of toxicity (also see Fenical and Harvell, 1988; Harvell and Fenical, 1989; Harvell et al., 1989) and vice versa. There is, however, measurable variance in this relationship, and the relationship only becomes apparent at high levels of taxonomic resolution.

ADAPTATIONS TO INTERSPECIFIC COMPETITION FOR SPACE

Competition between Alcyonaceans and Scleractinians. The ecological and evolutionary success of soft corals in the Indo-Pacific is evidence of the fact that they are effective competitors for space (see Jackson, 1977). Unlike scleractinian corals, however, alcyonaceans possess neither stenoteles (penetrant nematocysts; Barnes, 1974), sweeper tentacles (Richardson et al., 1979), or similar specialized anticompetitor defense apparati. Some soft corals (e.g., *Sinularia flexibilis* Quoy and Gaimard and various *Sarcophyton* spp.), however, possess particularly high concentrations of terpenoid toxins (flexibilide, FLX; Kazlauskas et al., 1978; and sarcophytoxide, SX, Tursch, 1976; Bowden et al., 1978a), accounting for up to 1% of their dry weight (Bowden et al., 1980).

Field experiments have demonstrated that some soft coral terpenoids cause tissue necrosis and mortality in scleractinian corals without contact. Investigations were made into three species of soft corals (*Lobophyton pauciflorum* Ehrenberg, *Sinularia pavida* Tixier-Durivault, and *Xenia* sp. affin. *danae*) competing for space with two scleractinian species (*Porites andrewsi* Vaughan and *Pavona cactus* Forskal). It was found that the occurrence and efficacy of allelopathic effects of soft corals on scleractinians varies species specifically between soft corals. Also, scleractinian corals vary in their susceptibility to these detrimental effects.

The involvement of terpenoids in this allelopathic interaction has been confirmed in laboratory studies. Pure terpenoids (FLX and FN-45) cause 100% mortality in two scleractinians (*Acropora formosa* Dana and *Porites andrewsi*) at concentrations as low as 5–10 ppm (Coll and Sammarco, 1983). But, again, the response curves vary species specifically with respect to both the toxin and the target species.

Experiments have also confirmed that terpenoids are present in the water column around the soft corals. A submersible sampling apparatus with a reversed-phase Sep-Pak was placed around undisturbed colonies of *Sinularia flexibilis* and *Sarcophyton crassocaule* in the field. Ambient concentrations of 1–5 ppm of the toxic diterpenes FLX and SX, respectively (Coll et al., 1982a).

Thus, the secondary metabolites present in soft corals can be transmitted as allelopathic agents, most likely accumulating in the lipid-rich tissues of neighboring organisms such as scleractinian corals.

Not all species with allelopathic capabilities, however, export their toxins (also see Gerhart, 1986). Field experiments examining similar interactions between three different soft corals (*Sarcophyton ehrenbergi* Maranzella, *Nepthea brassica* Kukenthal, and *Capnella lacertiliensis* MacFayden and Forskal) and the same two species of scleractinians examined earlier (*Pavona cactus* and *Porites andrewsi*) revealed that the highest degree of mortality in the latter occurred in this case as a result of contact (Sammarco et al., 1985). The amount of toxin released appears to be low in some soft corals, despite high concentrations of terpenes in the tissues (Sammarco et al., 1985; Bowden et al., 1978a, 1979).

Other adaptations can assist soft corals in competition for space. They may either complement the allelopathic capabilities of the octocoral or, on the other hand, obviate the need for chemical defense. Certain soft corals secrete a polysaccharide layer (cuticle) in the vicinity of the scleractinian, protecting them from damage from stinging nematocysts (Sammarco et al., 1985). In one case, this allows a soft coral (*Nephthea brassica*) to attach to the surface of a living scleractinian coral (*Acropora hyacinthus* Dana) and grow across it (La Barre and Coll, 1982).

Interspecific Competition between Alcyonaceans: Chemical, Behavioral, and Developmental Responses. Although competitive interactions between alcyonaceans and scleractinians are commonly observed in the field, similar interactions between alcyonaceans themselves appear to be infrequent. Tissue necrosis can be experimentally induced in interspecific interactions between alcyonaceans (*Alcyonium molle* Dean, *Sinularia flexibilis*, and *Nepthea brassica*) in both contact and noncontact situations (La Barre et al., 1986), but the response is a short-term one (on the order of weeks), decreasing in intensity with time. By shifting hydrostatic pressure within the colony, the organism can bend to avoid contact with its neighbor. Also, in the longer term (6–12 months), colonies move from their neighbors through directed growth and establish something akin to an individual distance (Conder, 1949) from their neighbors, thereby reducing the frequency of further interaction.

Growth and movement responses like these help to explain the paucity of interactions between alcyonaceans observed to occur naturally in the field. In fact, observed soft coral distribution patterns may be partially explained by the outcomes of historical competitive interactions. These interactions add another dimension to adaptations that have evolved in this group in response to competition for space.

OTHER ACTIONS OF SECONDARY METABOLITES

Antifouling. Although alcyonacean corals are generally characterized by the absence of fouling epibiota (e.g., bryozoans, filamentous algae, sessile polychaetes, etc.), fouling has occasionally been observed (Patton, 1972; Hartnoll, 1975; Morse et al., 1977; Goldberg and Makemson, 1981; Coll et al., 1987). One mechanism by which soft corals maintain their clean surfaces is the secretion of mucous sheets that are sloughed off. These sheets often harbor microalgae, which occasionally attach themselves to the colony in a more permanent manner. Slow release of terpenes from the colony may ultimately assist in their removal. This premise is supported by the finding that a number of terpenes derived from soft corals inhibit the growth of filamentous algae in unialgal culture (Coll et al., 1987; also see Targett et al., 1983) and prevent settlements of barnacle larvae (Standing et al., 1982; Rittschof et al., 1985, 1986, 1988; Keifer et al., 1986; Gerhart et al., 1988). Antifouling represents another ecological role of terpenes in the Alcyonacea.

Reproduction. Recent studies have opened questions about potential roles for secondary compounds in the reproduction of alcyonacean corals. In common with many scleractinian corals, Great Barrier Reef alcyonaceans have a mass spawning period during the early summer months (Babcock et al., 1986; Alino, work in progress). Many species are dioecious, releasing eggs and sperm simultaneously. The terpene composition of eggs released from several alcyonacean species is, in some cases, different from that of the tissue of the parent colony (Bowden et al., 1985; Coll et al., 1986, 1988). Thus far, compounds present in the eggs have not been shown to play a role in defense against planktonic or pelagic parasites or predators (P. Alino, work in progress), nor do they possess antibiotic properties against a range of marine bacteria.

Sperm chemotaxis has been demonstrated to occur in several species, but the chemicals responsible for this do not appear to be the egg-specific terpenoids (Coll et al., 1987). Although eggs may be present in the parent organism for up to 23 months, synthesis of terpenes within the eggs occurs only during the month immediately preceding spawning (Coll et al., 1988). The question as to whether these terpenes play some role in egg release remains open.

DISCUSSION AND CONCLUSIONS

Although secondary metabolites, particularly terpenes, occur commonly with the Alcyonacea, they exhibit high variability in their distribution. These terpenes may play multiple ecological roles. Although these may include anti-

predator adaptations, there does not appear to be any direct correlation between ichthyotoxicity and feeding deterrence. Other classes of molecules may well be more effective in this capacity.

Ichthyotoxicity is negatively correlated with morphological antipredator characteristics, but the association is only detectable at a high level of taxonomic resolution (e.g., genus or perhaps family) and only when a small number of morphological traits clearly related to predator defense are considered.

Many selection forces may operate differentially on each of the characteristics considered here, be they chemical or morphological. At a high level of taxonomic resolution, an adaptive response to predation, whether toxinological or morphological, is more readily discerned, and the range of variability in such a response is more restricted. At too low a level of resolution (e.g., across families), such responses in the same characteristics are easily counterbalanced and blurred by concurrent adaptations to other selection forces (Table 1). Colony morphology also evolves in response to such factors as food capture (Lewis, 1982), resistance to physical environmental stress (e.g., Fishelson, 1970; Dinesen, 1983), competition for space (Jackson, 1977; La Barre and Coll, 1982; Sammarco et al., 1983, 1985; La Barre et al., 1986a), optimization of photoreception by endosymbiotic zooxanthellae (e.g., Dustan, 1975), resistance to siltation (Fishelson, 1970; Schuhmacher, 1975; Dinesen, 1983), etc. Any one of these factors may act as a strong selection force in one species but becomes less important in others (Sammarco et al., 1987; Sammarco and Coll, 1988). Thus, an association between any two antipredator adaptations may be obscured by one or more other adaptations performing the same function.

Secondary metabolites play a complex role in competition for space between alcyonaceans and scleractinians. Concentrations of terpenoid compounds vary species specifically within the Alcyonacea as does the efficacy of the compounds against scleractinians. Some compounds are released more readily by some species than by others; some competitors are less susceptible to a given compound than others; and survival of competitors may be enhanced by other structural or behavioral anticompetitor defense adaptations. The roles that terpenoids play in alcyonacean soft corals are highly sophisticated, and their integration with other functionally related adaptations must be considered if one is attempting to explain variance in their distribution.

With respect to competition for space, the Alcyonacea exhibit a variety of chemical, developmental, and behavioral adaptations that are conplementary in function. Functional adaptations, whether chemical, morphological, behavioral, or developmental, are also highly species specific in their effectiveness. The presence of a toxic secondary metabolite is not a good indicator of an allelopathic agent nor does it necessarily imply any function in competition for space (Sammarco et al., 1985). The terpenes occurring in these organisms func-

TABLE 1. ANTIPREDATOR FUNCTIONS OF CHARACTERISTICS IN ALCYONACEAN SOFT CORALS[a]

Characteristic	Trait
Chemical	Ichthyotoxicity[1,2]
	Effects on predators
	Feeding deterrence[1,3]
	Olfaction
	Palatability
	Emetic response
	Synergistic effects (positive or negative) with other classes of compounds
Morphological[1,4]	Soft coral traits
	Microarmament of individual polyps
	Armament of polypary
	Mineralization of coenenchyme
	Sclerite sharpness?
	Color?
	Consistency of tissue?
	Colony flexibility?
	Growth form?
	Encrusting
	Stoloniferous
	Branching
Behavioral/physiological[1,4]	Polyp retractility
	Mucus secretion

[a] Evidence for functions determined through experimentation or observations; "?" denotes function yet to be confirmed. Superscripts refer to the following references: (1) La Barre, 1984; (2) Coll et al., 1982b; Coll and Sammarco, 1983; unpublished data; (3) La Barre et al., 1986b; (4) Sammarco et al., 1987.

tion in many different ways, depending on the soft coral species in question and the set of species—predators and competitors—with which it has interacted through evolutionary time. Each soft coral species has evolved in response to its own set of primary selective pressures. This has resulted in a given molecule derived from the same class of secondary compounds—the terpenes—serving different functions in different species.

Chemical characteristics functioning in competitor defense may or may not act in concert with other morphological or behavioral adaptations that serve a similar function (Table 2). In addition, these same morphological or behavioral characters may have other secondary or even primary functions that have evolved

TABLE 2. ANTICOMPETITOR CHARACTERISTICS IN ALCYONACEAN SOFT CORALS[a]

Characteristic	Trait
Chemical	Effects on Competitors[1,5–7]
	Allelopathic capabilities
	Contact
	Noncontact (waterborne)
	Growth inhibition/stunting
	Bleaching (expulsion of zooxanthellae)
	Induction of neoplasms
Morphological	Soft coral traits
	Sclerite density?
	Sclerite sharpness/abrasion?
	Basal polysaccharide cuticle[1,6,7]
	Colony flexibility[1,6,7]
Behavioral/physiological	Polyp retractility?
	Mucus secretion?
	Avoidance response (bending)[1,8]
	Movement (through growth)[1,8]

[a] "?" denotes function yet to be confirmed. Superscripts refer to the following references: (1–4) see Table 1; (5) Sammarco et al., 1983; Coll and Sammarco, 1983; (6) Sammarco et al., 1985; (7) La Barre and Coll, 1982.

in response to other selective forces (Table 1). The relative intensity of these selective forces varies greatly between species and thus, once again, the predictability of the occurrence of a toxin in a given alcyonacean, its function, and its association—negative or positive—with other functionally related adaptations decreases with further investigation.

TABLE 3. ANTIFOULING FUNCTIONS OF CHARACTERS IN ALCYONACEAN SOFT CORALS[a]

Characteristic	Trait
Chemical	Effects of epibiota
	Growth inhibition[9]
	Antibacterial activity[10]
	Antifungal activity[10]
	Antiviral activity[10]
Morphological	Soft coral traits
	Colony flexibility?
	(inhibition of colonization or attachment)
Behavioral/physiological	Mucus sheet formation[9]
	Ciliary action?

[a] "?" denotes function yet to be confirmed. Superscripts refer to following references: (1–7) see Tables 1 and 2; (8) Sammarco et al., 1985; (9) Coll et al., 1987; (10) Rinehart et al., 1981.

It is also possible that terpenes play a role in antifouling and enhancement of reproductive success, although their specific role(s) in the latter remains to be determined. Even these adaptations are tied to specific physiological or behavioral adaptations, and these latter adaptations themselves may have multiple roles (Tables 3 and 4). There may also be other functions that have not yet been considered, such as negative or positive settlement or association cues for larvae (sensu Rittschof et al., 1985; Morse et al., 1979, respectively).

Secondary compounds such as terpenes in alcyonaceans may play a role in any of these ecological processes, but the mere presence of the compound does not imply any particular role. It may play one or more different ecological roles

TABLE 4. FUNCTIONS OF CHARACTERISTICS IN ALCYONACEAN SOFT CORALS THAT ENHANCE REPRODUCTIVE SUCCESS[a]

Characteristic	Trait
Chemical	Toxic Effects
	Allelopathy[11]
	(increase success of larval settlement)
	Predator deterrents
	Eggs?[12,13]
	Whole colony during most fecund period?[12,13]
	Nontoxic effects
	Chemotaxis?[14]
	Sperm attractant?[14]
	Associate settlement cues?
	Spawning release factor?[13]
Morphological	Soft coral traits
	Growth form
	Encrusting
	Stoloniferous
	Branching
Behavioral/physiological	Surface brooding in mucus sheets[15]
	Synchronous spawning of gametes/ "predator swamping"—eggs[16]
	Nocturnal spawning[16]
	Brooding[16]
	Buoyancy of eggs and planulae (lipid content)[12,13,16]
	Taxis, planular?
	Phototaxis?
	Geotaxis?
	Thigmotaxis?

[a] "?" denotes function yet to be confirmed. Superscripts refer to following references: (1–10) see Tables 1–3; (11) personal observation; unpub. data; (12) Bowden et al., 1985; Coll et al., 1986; (13) Coll et al., 1988; (14) Coll et al., 1987; (15) Z. Dinesen, personal communication; (16) P. Alino, work in progress.

within a single species and is often highly species specific in action. A priori prediction of a function for a given secondary metabolite in an alcyonacean is virtually impossible because of the multiplicity of potential roles it can play. In addition, the presence or absence of a particular morphological or behavioral trait is a poor indicator of the presence or absence of chemical adaptation, and vice versa. This is due to similar confounding effects and evolutionary tradeoffs that have occurred between traits performing similar functions.

In any one species, the role of a secondary compound is integrated with various other adaptations (morphological, behavioral, reproductive, etc.), but such integration is complex, subtle, and highly species specific. Thus, if predictable relationships are to be found between secondary chemical characters and other functionally related attributes in a set of organisms, this is most likely to occur at high levels of taxonomic resolution.

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CONTROL OF OLFACTORY-INDUCED BEHAVIOR IN ALFALFA SEED CHALCID (*Hymenoptera: Eurytomidae*) BY CELESTIAL LIGHT

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Abstract—The behavioral response of female alfalfa seed chalcids is dependent on the polarization of sky light when exposed to olfactory stimuli between 1100 and 1500 hr. Females made no orientation flights to or landings on eight unbaited targets, but when half the targets were baited with the host-plant odor of hexyl acetate, they did make orientation flights to and landed on baited targets. Female preference for baited targets disappeared when natural sky light was plane polarized (at right angles to natural sky light polarization at 1200 hr). When natural sky light was passed through a diffuser filter to completely depolarize the light, females made numerous orientation flights but displayed no preference for baited targets. Any alteration of the natural wavelengths of sky light between 345 and 425 nm with various Kodak Wratten filters that excluded certain wavelengths produced similar results wherein females had no preference for baited or unbaited targets. The hypothesis that chalcid response was controlled by the polarization characteristics of sky light was confirmed when females again preferred baited targets in tests that were conducted 6 hr after sunrise, but they displayed no preference for baited targets when exposed to the mixture of polarized and nonpolarized light present in the sky 1–2 hr after sunrise or 1–2 hr before sunset. The possibility that chalcid response was due to a circadian rhythm was eliminated by exposure of insects to different sequences of light-dark regimes prior to the olfactory test.

Key Words—Alfalfa seed chalcid, *Bruchophagus roddi*, Hymenoptera, *Eurytomidae*, Chalcidae, olfactory behavior, polarization vision, insect behavior.

INTRODUCTION

The classic work of von Frisch (1949) showed that honeybees (*Apis mellifera* L.) use the celestial pattern of polarized light as a compass. They also have trichromatic color vision composed of ultraviolet (345 nm), blue (440 nm), and green (550 nm) with a predominance of blue receptors (Menzel, 1975). Many herbivorous species of insects respond to the pattern, dimension, and spectral quality of plants. Spectral reflectance of plant buds, bracts, blossoms, seeds, fruits, stems, twigs, and thorns may be similar or different in reflectance (Prokopy and Owens, 1983). How phytophagous species perceive and use polarized light to assess plants is unknown for most species.

Polarization vision, like color vision, is three-dimensional but for different reasons and requires three types of receptors (Bernard and Wehner, 1977). Polarizational states are analogous to colors that can be described by intensity, angle of polarization (*e* vector orientation), and degree of polarization. The percentage of polarized sky light increases from the sun, where it is zero, to an angular distance of 90° from the sun (Wehner and Rossel, 1985). Also, a patch of sky is characterized not only by the degree of polarization, but also by its spectral composition. Those portions of the sky that exhibit maximum polarization also have the highest content of short-wavelength light. These characteristics of celestial light may influence the way insects perceive and evaluate plant stimuli.

The effect of polarized light has not been investigated for most phytophagous insects, but certain components of light are known to affect host-finding. For example, the fruit fly, *Rhagoletis pomonella* (Walsh), detects its host fruit by intensity contrast of dark fruit against a bright background of light transmitted through foliage (Owens and Prokopy 1987). One also cannot rule out an interaction of light and odor perception in phytophagous insects (Visser, 1986), as occurred in tests with tsetse flies (*Glossina* spp.) (Green, 1986).

During flight and host-finding tests with alfalfa seed chalcids, *Bruchophagous roddi* (Gussakovsky), females responded differently to the same olfactory stimuli in the laboratory and greenhouse. Reported herein are tests with different types of light that changed the way female chalcids responded to olfactory stimuli.

METHODS AND MATERIALS

Experimental insects were reared in the laboratory in controlled environment chambers as described elsewhere (Kamm and Buttery, 1986). Females were collected the day of emergence and put into vials (95 × 24 mm diam.) provisioned with a 10% solution of sugar water, 25 per vial. The rearing regime

was 16 : 8 light–dark at 24°C with lights programmed to begin at sunrise. Once in vials, females were exposed to 30°C in one of the following pretest light regimes of 16 : 8 light–dark for 48–72 hr where the lights in the chambers came on; 6 hr before sunrise, 6 hr after sunrise, or sunrise. The latter was used unless specified otherwise.

To obtain a relatively uniform distribution of light, the bioassay chamber (Kamm, 1988) was used inside a large plywood box (1.2 × 1.2 × 2.4 m long) with an open top (Figure 1). Parallel observation ports (5 × 65 cm) were cut in the sides of the box, positioned 68 cm from the top and 60 cm from the ends. The observer viewed the tests from under black shading cloth attached to the box so light did not enter through these ports. The chamber was used in a greenhouse room, half of which had a thin layer of shading compound to reduce the intensity of direct sunlight and avoid heat build-up inside the greenhouse. Otherwise, test insects had a 160° view of the sky from east to west during bioassays. No plants were grown in the bioassay greenhouse. One end of the plywood box was fitted with an intake fan and the other with an exhaust fan mounted in openings 20 cm in diam. These fans were used to move fresh air through the chamber and to exhaust test odors out of the greenhouse after a test.

Test choices consisted of eight wax targets (previously illustrated; Kamm, 1989) deployed in the chamber in two rows and spaced 17 cm apart. Hexyl acetate, a known host-plant attractant (Kamm and Buttery, 1986), was used as bait by dispensing 25 μl of 0.1% hexyl acetate in hexane onto a piece of cotton

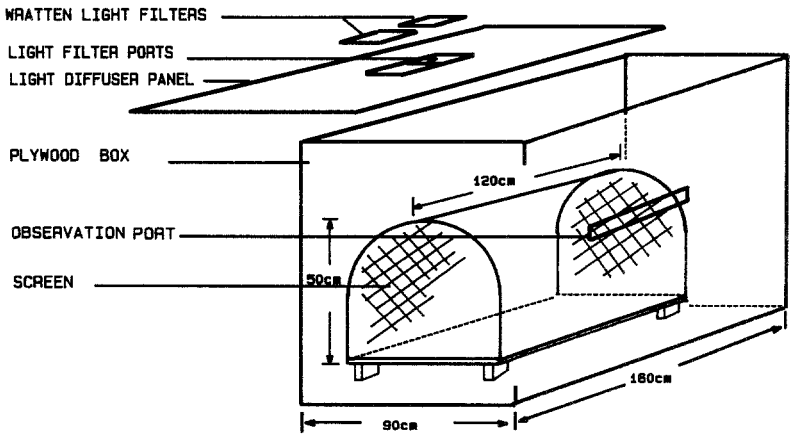

FIG. 1. Diagram of bioassay chamber inside a large plywood box (painted white). Either the light diffuser panel (shown) or the polarized filter (not shown) was taped to the top of the box to conduct a test. The Kodak Wratten filters were taped over the ports cut in the diffuser panel.

dental roll 1 cm long. Blank targets had similar dental rolls impregnated with 25 μl of hexane. After the solvent evaporated, the rolls were suspended and hidden inside the wax target as described (Kamm, 1989). Half the targets were baited with hexyl acetate and half with hexane (blank). Test odors dispersed through the chamber by diffusion to form a concentration gradient around each source of odor.

Filters were used to obtain certain types of light. A light diffuser (No. 444 luminous panel, Armstrong Corp., Seattle, Washington) was used to completely depolarize incoming light. Two adjacent ports (110 × 110 mm) were cut out of the center of the 61 × 122-cm diffuser panel so either one or two Kodak Wratten light filters could be used simultaneously. When one filter was used, the other port was covered with a piece of the diffuser panel. The polaroid filter (HN 35 neutral color, 48 × 128 cm long) was a cellulose acetate butyrate linear polarizer obtained from Polaroid Corp. (Norwood, Massachusetts). This filter was used in place of the light diffuser panel shown in Figure 1. Both the Polaroid filter and diffuser panel were taped to appropriate-sized tagboard frames that were then secured to the plywood box to exclude all other light. Kodak Wratten filters 0, 2B, 2E, and 18A (125 × 125 mm) were used in combination with the diffuser filter as illustrated (Figure 1). Each filter excluded specific wavelengths and permitted others to pass through the filter. All filters were gelatin except 18A, which was glass and permitted entry of specific wavelengths of light between 345 and 355 nm. Only four targets (two of which had hexyl acetate) were used in tests with Kodak filters because of the relatively small area beneath the filters. The light intensity was measured with a light meter prior to a test and ranged from 28,000 to 52,000 lx inside the bioassay chambers.

To conduct a test, eight vials (95 × 24 mm diam.) of 25 females were placed upright in the bioassay chamber and the stoppers removed so females could exit and disperse within the chamber. After the insects had acclimated to the chamber environment (29°C) for 30 min, the targets were introduced and the insects observed for 1 hr. This procedure was replicated three times for each test. The observer monitored only that air space within 10 cm of the array of targets (delineated by parallel lines along the side of the chamber) for the number of orientation flights, orientation landings, and direct landings. Females were not removed after an orientation flight because they tend to stay where they land 2–10 min and then fly off in a different flight mode. Orientation flights were considered terminated when the insect lost interest in the stimuli and flew out of the monitoring zone or landed on the stimuli. How the observer distinguished these behaviors is described elsewhere (Kamm, 1989). Briefly, the test criteria were: (1) Orientation flight—chalcid slowly flew around and/or directly toward specific targets with the antennae thrust forward while the abdomen was held vertically. These flights usually occurred within 10 cm of the array of test

stimuli and were often punctuated by hovering flight when close to the target. (2) Orientation landing—chalcid hovered close to or circled the target and then gently landed on it. (3) Direct landing—chalcid flew swiftly to and landed forcefully on a target without close-range evaluation. χ^2 analysis was used to analyze all test data with the hypothesis that the ratio of chalcids that responded to baited and unbaited targets was 1 : 1. No analysis was done in tests where fewer than six insects responded in a given category.

RESULTS AND DISCUSSION

When females were exposed to a randomized array of baited and unbaited targets in unfiltered naturally polarized sky light, they preferred targets baited with hexyl acetate to unbaited targets (blank) as indicated by the numerous orientation flights and landings (Figure 2A). When exposed to only unbaited targets, females made no orientation flights and landings but instead made numerous direct landings on the targets (Figure 2B). These tests clearly indicated that the odor stimulus was the factor that elicited an orientation response directed toward the targets, a process that commonly occurs in other insects (Kennedy, 1977). Similar results were obtained when a Polaroid filter was used but aligned (visually positioned to permit entry of maximum natural polarized

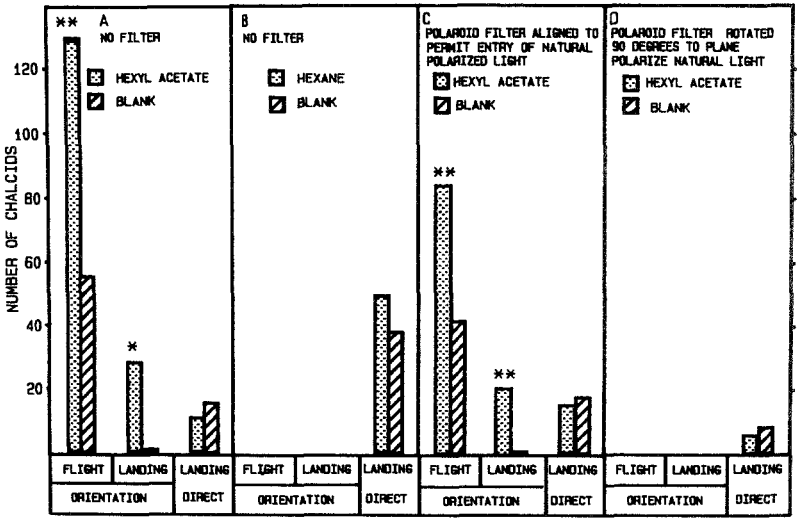

FIG. 2. Total number of female alfalfa seed chalcids that responded when exposed to olfactory stimuli in unfiltered sky light or sky light passed through a polaroid filter between 1100 and 1500 hr. $*P < 0.05$; $**P < 0.01$, χ^2.

light) so it did not alter the natural polarization of sky light (Figure 2C). However, when the Polaroid filter was rotated 90°, it blocked certain light vibrations and permitted entry of polarized light aligned only in a single plane. Then the chalcids made no orientation flights to or orientation landings on targets (Figure 2D). Females clearly required the celestial pattern of polarized light to respond to the hexyl acetate bait. Although light intensity was greatly reduced by the Polaroid filter, it was the same in tests C and D of Figure 2.

A different approach was used to confirm the previous results by using a light diffuser (Figure 1) that completely depolarized the sky light entering the bioassay chamber. First, evidence was obtained that light passing through the diffuser was depolarized by placing it between two Polaroid sheets. Light was transmitted equally when both Polaroid filters were aligned and also when the top filter was rotated 90°. This indicated the light was completely depolarized (a uniform random scattering of light) because the latter arrangement would not have transmitted light had light been polarized in a single plane. Light was not transmitted by the combination of Polaroid filters without the diffuser between them when the top filter was rotated 90°. When the diffuser was used to depolarize natural sky light, females made numerous orientation flights but displayed no preference for baited targets (Figure 3A). Thus, females again displayed no olfactory preference when the natural polarization of light was altered using a procedure other than Polaroid filters. When unfiltered light entered through ports in the center of the diffuser, chalcids regained their preference for baited targets (Figure 3B). Similar results were obtained when Kodak Wratten filter 0 (essentially unfiltered light through gelatin) was used in the port (Figure 3C). However, when Kodak filters 2B, 2E, and 18A were used alone and in different combinations to exclude specific wavelengths of light between 345 and 425 nm, females displayed no preference for targets baited with hexyl acetate (Figure 3D–H). This indicated that the natural color of light also plays an important role in the olfactory behavior of chalcids.

Because the degree and angle of polarization changes as the earth changes position relative to the sun, tests were made to determine how such gradients influence response of females to olfactory stimuli. When females were exposed to a regimen of 16:8 light–dark where the photophase began 1 hr before sunrise, females displayed no olfactory preference when the test began 1 or 10 hr after sunrise, but they preferred the baited targets when the test began 5 hr after sunrise (Figure 4A–C). The chalcids may have responded because they were trained to a lights-on signal that evoked a circadian response instead of a response to the natural polarization of light. Thus, to determine whether or not the response was circadian, females were exposed to the same 16:8 light–dark regimen as in previous tests but the light period began 5 hr before sunrise. Again females displayed no olfactory preference when the test began 1 hr after sunrise but preferred baited targets when the test began 5 hr after sunrise (Figure 4D

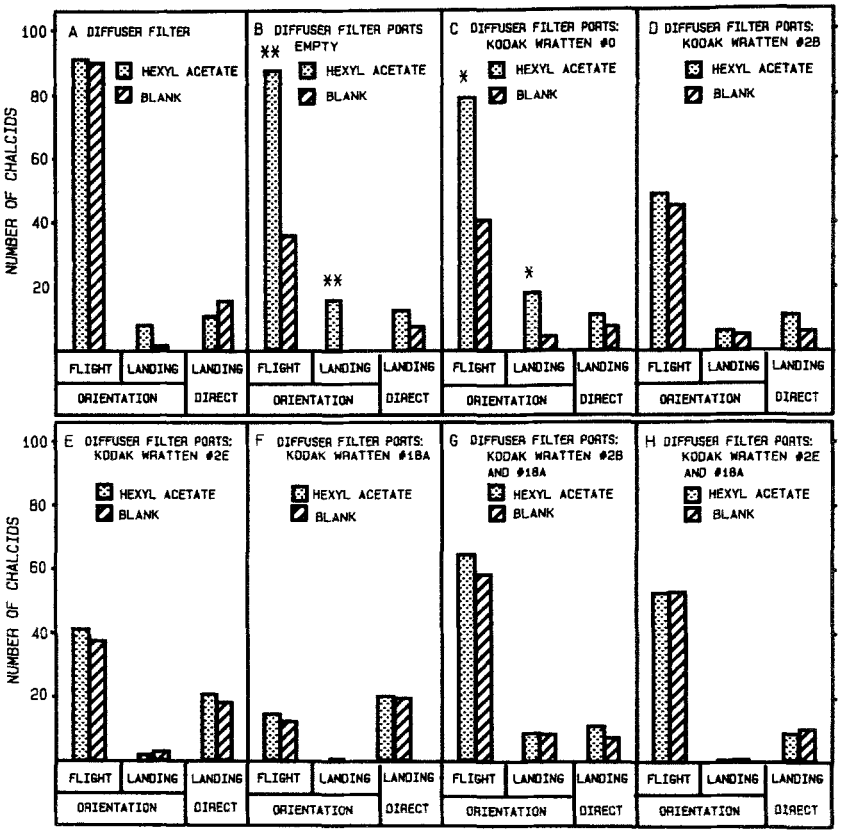

FIG. 3. Total number of alfalfa seed chalcids that responded to olfactory stimuli when sky light was completely randomized by a diffuser filter used alone and in combination with Kodak Wratten filters. $^{*}P < 0.05$; $^{**}P < 0.01$, χ^2.

and E). In yet another test, females were exposed to 16:8 light–dark where the dark period began 4 hr before and continued 4 hr after sunrise. Females were exposed to the targets 2 hr after the beginning of the light period (6 hr after sunrise), and again females preferred baited targets (Figure 4F). Thus, a circadian response was not the mechanism that influenced olfactory response in chalcids.

In summary, chalcids made inflight maneuvers toward and landed on targets baited with hexyl acetate when tests were conducted under natural wavelengths and polarization of sky light. Any alteration of these parameters eliminated female preference for baited targets. As Dethier (1982) pointed out, the use of behavior as a criterion of recognition does not necessarily mean

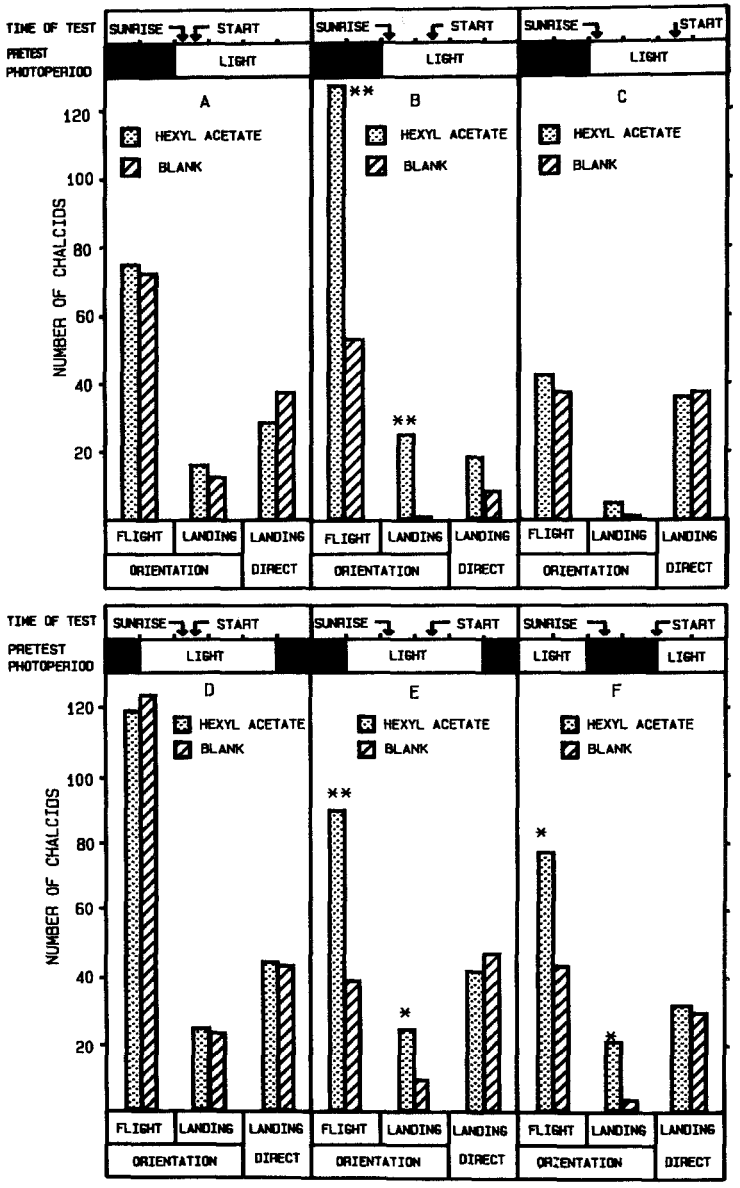

FIG. 4. Total number of alfalfa seed chalcids that responded when exposed to different light-dark regimens prior to testing female response to olfactory stimuli in unfiltered natural sky light when tests began 1, 5, or 10 hr after sunrise. $*P < 0.05$; $**P < 0.01$, χ^2.

absence of recognition when the behavior is absent. Nevertheless, alteration of natural light changed the way chalcids responded to olfactory cues during flight. Previously (Kamm and Buttery, 1986), the absence of natural light and the use of artificial light in no-choice tests did not prevent chalcids from identifying hexyl acetate as an active component of alfalfa. However, these tests did not consider inflight evaluation of the chemical stimuli. Here, natural light was essential for chalcids to evaluate visual and olfactory stimuli during flight if they were to display a preference when confronted with a choice. Important behaviors of other insects may be inhibited when bioassays are conducted in the laboratory without the natural color and/or the polarized gradient of sky light. In general, it is difficult to stimulate insects to fly upwind in response to plant odors (Finch, 1986), and natural light may be required to induce such behavior in some insects.

The mechanism chalcids use to perceive light cues is probably similar to that of honeybees, where the spatial arrangements of ultraviolet receptors in the eye read the point of maximum polarization in the sky (Wehner and Possel, 1985). Direct sunlight contains less than 10% ultraviolet radiation, while scattered sky light may have up to four times more. Chalcids failed to display a preference for baited targets when exposed to a light spectrum that excluded ultraviolet light below 390 nm using Kodak Wratten filter 2B. Ultraviolet light functions as a primary color for many insects (Brunner and Labhart, 1987; Kevan and Baker, 1983). The chalcid may use the color of light and the degree and angle of polarization to determine the time of day it engages in olfactory and visual search behavior. Although temperature clearly plays an important role in chalcid flight (Strong et al., 1963), the natural polarization of light may confine olfactory activity to midday in areas where night temperatures stay above 21°C.

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CORRELATION OF PHENOLIC ACID CONTENT OF MAIZE TO RESISTANCE TO *Sitophilus zeamais*, THE MAIZE WEEVIL, IN CIMMYT'S COLLECTIONS

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Abstract—The (*E*)-ferulic acid content of the grain of nine populations of land races of maize derived from CIMMYT's collections was found to be negatively correlated to susceptibility characteristics towards the maize weevil *Sitophilus zeamais*. Correlation coefficients for six susceptibility parameters and (*E*)-ferulic acid content were significant and ranged from −0.58 to −0.79. A multiple regression analysis by the SAS forward procedure using the primary seed characteristics associated with susceptibility indicated that the ferulic acid content was the only significant factor in explaining variation in at least two susceptibility parameters: the Dobie index and adult preference. In 15 CIMMYT pools, correlations between four susceptibility parameters and (*E*)-ferulic acid content were also significant (−0.76 to −0.81). The results suggest that phenolic acid content is a leading indicator of grain resistance or susceptibility to insects and may represent a newly identified mechanism of resistance.

Key Words—Phenolic acids, resistance, susceptibility, maize, maize weevil, *Sitophilus zeamais*, Coleoptera, Curculionidae.

INTRODUCTION

Sitophilus zeamais Motsch., the maize weevil, is both a preharvest and a postharvest pest of maize. Infestations can occur in the field while the grain is still in the husk or in storage after the maize is shelled. Races of maize vary greatly

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in susceptibility to attack by the corn weevil (Eden, 1952; Dobie, 1977; Serratos et al., 1987). The husk cover does provide some protection from attack (Dobie, 1977), but other factors contribute to the resistance of shelled grains.

In maize, variation in seed characteristics related to nutritional quality such as sugar and starch content, protein, fat, oil, hardness, and ash have been assessed for a possible role in susceptibility to either *S. zeamais* or *S. oryzae* (Dobie, 1977; Eden, 1952; Singh and McCain, 1963; Gomez et al., 1982, 1983; McCain and Eden, 1965). Many of these factors are correlated in some way with susceptibility.

A previous study by this group using two susceptible and two resistant maize populations suggested a possible role for maize phenolics as deterrents to *S. zeamais* (Serratos et al., 1987). The resistant maize populations had significantly higher phenolic acid levels in the pericarp than susceptible varieties, and feeding studies with maize extracts and pure phenolic acids added to artificial diets suggested the antifeedant effects of these compounds.

The purpose of the present study was to investigate the role of phenolic acids in maize within the context of other nutritional and physical factors that are known to affect susceptibility of grain to maize weevil. Two sets of maize from CIMMYT's (International Centre for Wheat and Maize Improvement, El Batan, Mexico) collections were studied. A set of land races was examined because in our previous study (Fortier et al., 1982), two land races were identified as having exceptionally high resistance and because land races have a high degree of variability useful for determining factors associated with resistance. In addition, 15 CIMMYT pools were examined to determine if the results extended to modern improved germplasm.

Susceptibility of maize populations to maize weevil was assessed by standardized tests for the number of oviposition sites, number of progeny produced, consumption by adults and a related stability parameter, an adult preference test (choice), and a life-cycle index of susceptibility (the Dobie index). Initially, hardness, the principal phenolic acid [(*E*)-ferulic acid], sugar and protein contents were examined in the land races. A more detailed analysis of individual maize phenolics [(*Z*)-ferulic, *p*-coumaric and sinapic acids, Figure 1] was carried out with the pools.

METHODS AND MATERIALS

Plant Material

Nine collections of Mexican land races and 15 pools from CIMMYT's collection were multiplied at El Batan, Tlaltizapan, and Poza Rica field sites from germ-plasm bank material held at CIMMYT (Table 1). The Mexican land races represent rare germ plasm of maize described by Wellhausen et al. (1951).

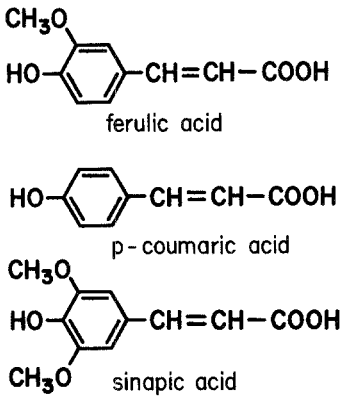

FIG. 1. Phenolic acids of maize grain.

TABLE 1. GERM PLASM (LAND RACES AND CIMMYT POOLS) ANALYZED IN STUDY

Race or Pool	CIMMYT collection
Arrocillo Amarillo[a]	Puebla 463, Puebla 537
Cacahuacintle[a]	Mexico 212
Chalqueno[a]	Mexico 208
Conico[a]	Mexico 182, Mexico 461
Oloton[a]	Chiapas 218
Palomero Toluqueno[a]	Mexico 5, Mexico 55
Pool 9 HLWD[b]	
Pool 15 TEWF	
Pool 16 TEWD	
Pool 17 TEYF	
Pool 18 TEYD	
Pool 19 TIWF	
Pool 20 TIWF	
Pool 21 TIYF	
Pool 22 TIWD	
Pool 23 TIWF	
Pool 24 TIWD	
Pool 25 TLYF	
Pool 26 TLYD	
Pool 27 SEWF	
Pool 28 SEWD	

[a] Land race.
[b] Abbreviations: W = white, Y = yellow, F = flint, D = dent, H = highland, T = tropical, S = subtropical, L = late, I = intermediate, E = early.

The chemical analyses were done on flours, and hardness was assessed using intact whole grains.

Assessment of Resistance

The following procedure was used with CIMMYT pools: cultures of *Sitophilus zeamais* were reared in 1-liter screened Mason jars and maintained in an environmental chamber at 27°C, 70% relative humidity, and 16-hr photoperiod on a diet of Ritchies Recleaned Corn, a locally available feed corn. Prior to use in the resistance tests, all corn was stored in a cold room at 4°C. Varieties to be tested were placed in 1.5-liter screened Mason jars and kept under the same conditions as *S. zeamais* for a minimum of six weeks before beginning resistance tests (Widstrom et al., 1978). After this time, average moisture content of each variety was determined by accurately weighing three 5-g samples of seed, taken randomly from different levels in the Mason jar. The samples were dried in an oven at 130°C for 16 hr and reweighed (Fortier et al., 1982).

Seed sample size and infestation of *S. zeamais* were determined following the recommendations of Widstrom et al. (1978): there was 1 g seed per adult weevil and more than the minimum of 20 weevils required to avoid sex determinations. All tests were carried out in the dark at 27°C, 70% relative humidity by placing the vials in a black cloth bag in the environment chamber. Ritchies Recleaned Corn and Cacahuacintle, a high-altitude, floury, Mexican corn susceptible to *S. zeamais* were used as controls (Van Der Schaaf et al., 1969; Dobie, 1977). A resistance test was performed on each variety plus controls using the following technique developed by Dobie (1977) with some modifications. For each variety, two subsamples of 200 seeds each (50+ g) were placed in screened 40-g plastic vials. Each sample was infested with 50 adult *S. zeamais* weevils, 0–7 days old. These first infestations were known as the conditioning replicates, and their purpose was to allow the weevils to adjust to any short-term affects. After seven days, moisture content of each variety was determined and adult mortality recorded. Two more samples of 200 seeds were counted, weighed, and placed in vials (test replicates). Surviving weevils from the conditioning replicates were transferred to the test replicates, and the test replicates were returned to the chamber for one more week. Adult mortality was again noted (only this mortality figure is reported in the results).

At the end of the second week, one test replicate of each variety was returned to the chamber to await emergence of progeny adults. Once emergence began, progeny adults were counted and removed daily for approximately 28 days. Upon termination of the test (no further emergence), frass was removed from the sample by blowing the seeds with a stream of compressed air before weighing. The mean number of progeny per 50 adults or 200 seeds represents the parameter "progeny." The second test replicate of each variety was placed

in the refrigerator after removal of the adult weevils in preparation for counting of egg plugs. Frass was removed from the samples prior to staining with 20 ppm berberine hydrochloride for counting (Milner et al., 1950). All 200 seeds were examined and egg plugs counted under UV light. Presence of stained egg plugs was confirmed by examining some seeds under a dissecting microscope. For each variety, the entire procedure was repeated four times and the mean number of eggs/50 adults (or 200 seeds) represents the parameter "egg number."

The Dobie (1977) index of susceptibility (I) was determined from the data where:

$$I = \ln \frac{F}{D} \times 100$$

and F = total number progeny adults and D = mean development time (days).

The methods for determining and other susceptibility parameters (stability factor based on rate of consumption, consumption of grain, choice test) are described in Serratos et al. (1988).

Determination of Phenolic Acid and Fatty Acid Content

Alkaline Hydrolysis (4 and 18 hr). The total content of the phenolic acids and fatty acids in 1 g of ground grain was hydrolyzed in 30 ml of 2 N sodium hydroxide in an enclosed flask in an atmosphere of nitrogen. The flask was placed on a shaker for 4 or 18 hr and subsequently acidified to pH 2.0 with 6 N hydrochloric acid. The slurry was centrifuged at 750g for 10 min, and the supernatant was decanted into a separatory funnel. The pellet was rinsed twice with water and centrifuged after each wash. All supernatants were combined, and the phenolic acids and fatty acids were extracted with 40 ml of ethyl acetate. The upper organic layer and the lower aqueous layer were collected separately. The aqueous layer was reextracted with 40 ml of ethyl acetate. The upper ethyl acetate layer from this second extraction was combined with the upper layer from the first extraction and extracted with 40 ml of water. The ethyl acetate layer from the final extraction, including any emulsion that formed between the upper and lower layers, was collected and dehydrated over sodium sulphate (anhydrous). The extract was dried in vacuo and the residue was redissolved in 3 ml of ethyl acetate.

Acid Hydrolysis. The acid hydrolysis procedure used was based on the method of Pussayanawin and Wetzel (1987). The flour was hydrolyzed in a test tube with 35 ml of 0.2 N sulfuric acid in a boiling water bath for 30 min. The tube was cooled under running water and 5 ml of a 2% (w/v) suspension of a fungal amylase (Sigma) in 2.5 M aqueous sodium acetate was added to digest the starch in the sample and to simplify subsequent procedures. The mixture

was incubated in a water bath at 55°C for 60 min with periodic agitation. The sample was centrifuged at 750*g* for 10 min. The supernatant was collected and the pellet was washed twice with water and centrifuged. The supernatants from all three centrifugations were combined and adjusted with water to a volume of 50 ml. The phenolic acids were extracted three times with 100 ml ethyl acetate. The upper (ethyl acetate) layers were combined and dehydrated over sodium sulphate (anhydrous). The extract was dried in vacuo and redissolved in 3.0 ml of ethyl acetate.

Separation by Gas Chromatography. To prepare the samples, all samples were filtered (0.45 μm filter) and an aliquot of 1 ml from each extract was dried under nitrogen. Hydroxybenzoic acid was used as an internal standard, and 20 μg was added to each sample and dried under nitrogen. The samples were derivatized prior to injection with *N*, *O*-bistrimethylsilyl acetamide (Serva) and diluted with methylene chloride.

The Vista Varian 6000 gas chromatography system with a flame ionization detector was used. One or five microliters were injected onto a 10-m bonded phase vitreous silica capillary column (ID 0.22 mm, OD 0.33 mm) with a flow rate of 3.0 ml/min. The initial oven temperature was 150°C. After 2 min, the oven temperature was increased at a rate of 10°C/min for 12 min and then held at 250°C for 3 min. The peaks were recorded on a Hewlett-Packard Integrator. The areas under the peaks were integrated and corrected for the volume of sample injected and dilution. The areas of the peaks for the individual components were divided by the area of the peak for the internal standard to correct for any variation in injection volume. The areas were converted to micrograms of compound according to a standard curve constructed using standards of (*E*)-ferulic acid and hydroxybenzoic acid. Phenolic acids and fatty acids were separated using this procedure.

Identification. Identification of the components was made by gas chromatography–mass spectrometry (GC-MS) and by comparison with standards. The GC-MS analysis was performed on derivatized samples and under similar chromatographic conditions to those used to obtain the quantitative data.

Determination of Hardness

The grain was equilibrated for six weeks in a growth chamber at 27°C and 70% relative humidity before determining hardness. The Instron system for determining hardness was made up of two parts. The testing system consisted of a model 4201 Instron (Canton, Massachusetts) with a strain-gauge indicator (Daythonic, model 3170, Dayton, Ohio) and a load cell of 0–1000 lb capacity (Strainsert model FLIU-35PKT, West Conshocken, Pennsylvania). The computer system consisted of an Apple II Plus microcomputer with a real-time clock

card, a video display monitor, a matrix printer (Apple dot matrix), and two disk drives. A description of the entire system and the program can be found in Buckley et al. (1984).

Each sample unit was tested at room temperature and force peak (newtons) was averaged per each variety. Since hardness is defined as the height of the force peak on the first compression cycle (first bite) (Borne, 1982), this measure was taken.

Determination of Protein and Sugar Content. Total sugar was analyzed by a modified Dubois method as described previously (Serratos et al., 1987) and protein in whole grain was estimated by a kjeldahl nitrogen analysis procedure using an automated kjeltec (Decator) analyzer and 6.25 as conversion factor.

RESULTS

Phenolic Extraction

A review of the literature suggested at least three major variations on phenolic acid extraction methodologies; 4-hr base hydrolysis (Sosulski et al., 1982), overnight base hydrolysis (Ribereau-Gayon, 1972), and a 30-min acid hydrolysis (Pussayanawin and Wetzel, 1987). As seen in Table 2 overnight (18-hr) base hydrolysis resulted in the highest recoveries of (*E*)-ferulic acid with 4-hr base hydrolysis providing near equivalent amounts. The acid hydrolysis pro-

TABLE 2. A COMPARISON OF RANGE OF PHENOLIC ACIDS AND FATTY ACID RELEASED FROM MAIZE GRAIN BY THREE DIFFERENT HYDROLYSIS METHODS

Type of extraction	Compound (μg/g flour)				
	(*Z*)-Ferulic	(*E*)-Ferulic	*p*-Coumaric	Sinapic	Palmitic
4-h base hydrolysis	28–64 (10)[1]	199–443 (10)	22–75 (10)	11–19 (10)	9–78 (10)
18-h base hydrolysis	52–698 (10)	113–1194 (10)	0–3 (1)	0–224 (6)	424–3223 (10)
Acid hydrolysis	0–47 (8)	37–416 (10)	0–3 (1)	0–17 (2)	4–82 (10)

[a] The numbers in parentheses are the numbers of samples in which the compound was detected.

duced very low values. Both overnight and acid hydrolysis were incompatible with accurate analysis of the minor phenolic acids [(*Z*)-ferulic acid, *p*-coumaric acid, and sinapic acid] because of either isomerization and release of interfering fatty acids by saponification (18-hr base hydrolysis) or extremely poor recoveries (acid hydrolysis). For these reasons, the 4-hr base hydrolysis was adopted as the best method for varietal comparisons of all phenolics.

Excellent separation of maize phenolics from extracts was obtained by a gas chromatographic method developed using a capillary column (Figure 2). (*E*)-Ferulic acid, (*Z*)-ferulic acid, *p*-coumaric acid, and sinapic acid as well as the fatty acid palmitic acid were identified by GC-MS.

Correlations in Land Races

Five physical and chemical parameters for nine collections of land races of maize are given in Table 3. Means of six susceptibility parameters for the same land races are reported elsewhere (Serratos, 1988). All the susceptibility parameters are highly ($r \geq 0.58$) and frequently ($r \geq 0.79$) and significantly ($P \leq 0.05$) correlated with each other (data not shown). In Table 4 simple correlations between some of these parameters are given. There was a signifi-

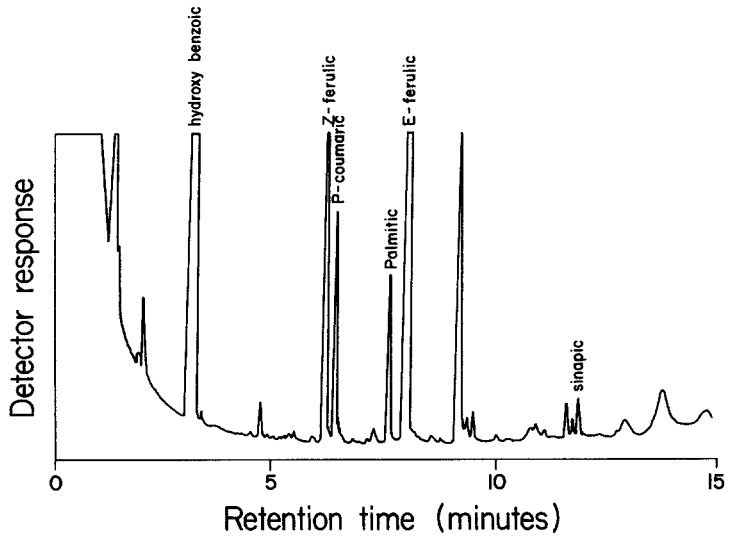

FIG. 2. GC separation of silated phenolic and fatty acids in maize grain extracts.

TABLE 3. PHYSICAL AND CHEMICAL DATA ON LAND RACES[a]

Collection	(*E*)-ferulic acid (μg/g)	Total sugar (mg/g)	Kjeldahl protein (%)	Instron hardness (N)	Palmitic acid (μg/g)
C218	394	15.9	10.9	83.5	5.97
M5	1438	14.3	13.4	105.4	31.09
M55	1517	16.4	12.3	130.5	22.13
M182	1085	20.0	12.1	62.9	6.38
M208	1411	13.2	11.7	80.8	8.73
M212	767	18.4	10.9	60.2	2.35
M461	1467	12.3	11.4	59.0	7.68
P463	1600	15.8	12.6	87.6	27.43
P537	1700	15.5	13.0	111.5	26.49

[a] Values are means of a minimum of four samples.

cant negative correlation between all the susceptibility parameters and the following physical chemical characteristics: (*E*)-ferulic acid, protein, or hardness ($r = -0.5$ to -0.9). Palmitic acid showed a negative correlation with the Dobie index.

In order to assess the significance of (*E*)-ferulic acid in susceptibility with respect to other physical–chemical parameters, a multiple regression was undertaken using the SAS forward selection procedure, entering only parameters with $P \leq 0.1$ into the model. The six susceptibility parameters were taken in turn as the independent variable, with the physical–chemical parameters as dependent variables. Multiple regression coefficients, r^2, F, and P values are given in Table 5. Ferulic acid is the only significant factor in explaining variation in the choice test (adult preference) and Dobie index (life-cycle index) while protein is significant for the other parameters (egg number, emergence, consumption, and stability parameter).

Correlations in CIMMYT Pools

Generally similar results were found in the correlations for the 15 CIMMYT pools. However, these were further analyzed for phenolic acid content [(*Z*)-ferulic, (*E*)-ferulic acid, *p*-coumaric acid, and sinapic acid] as measured by gas chromatography analysis (Table 6). Highest correlations ($r \geq -0.75$) were found between all susceptibility parameters and the content of the principal phenolic acid, (*E*)-ferulic acid (Table 7, Figure 3). These were highly signifi-

TABLE 4. CORRELATIONS BETWEEN SUSCEPTIBILITY PARAMETERS AND PHYSICAL–CHEMICAL CHARACTERISTICS IN LAND RACES[a]

Grain characteristic	Susceptibility parameters					
	Egg number	Progeny	Stability parameter	Adult choice	Dobie index	Consumption
(*E*)-Ferulic	−0.71**[a]	−0.58*	−0.62*	−0.79***	−0.77***	−0.63*
Hardness	−0.62*	−0.64*	−0.63**	−0.61*	−0.63**	−0.71**
Protein	−0.74**	−0.71**	−0.69**	−0.69**	−0.69**	−0.73**
Palmitic acid	−0.56	−0.61*	−0.31	−0.49	−0.66**	−0.55
Sugar	+0.23	+0.28	+0.30	+0.30	+0.02	+0.39

[a]Values are Pearson's correlation coefficient. ***$P \leqslant 0.01$; **$P \leqslant 0.05$; and *$P \leqslant 0.1$. All other values $P \geqslant 0.1$.

TABLE 5. PARTIAL COEFFICIENTS FOR REGRESSION OF SUSCEPTIBILITY AS A FUNCTION OF PHYSICAL–CHEMICAL PARAMETERS[a]

Susceptibility parameter	= Intercept	+ B_1 × (*E*)-ferulic acid	+ B_2 × protein	r^2	*F*/*P*
Dobie index	13.18	−0.00366		0.60	10.4/.01
Insects attracted (choice test)	60.32	−0.00331		0.63	11.7/.01
Stability parameter	4.61		− 0.300	0.48	6.3/.04
Progeny	175.8		−13.5	0.50	7.1/.03
Egg number	137.6		−10.3	0.56	8.9/.02
Consumption	3.10		− 0.189	0.53	8.0/.03

[a] A multiple regression analysis was undertaken with the SAS forward selection procedure and using the independent variables ferulic acid, protein, hardness, palmitic acid, sugar, and moisture. Only variables with a $P \leq 0.1$ were introduced into the model.

cant at $P \leq 0.01$. Correlations between the susceptibility parameters and the next most important phenolic acids, (*Z*)-ferulic acid and *p*-coumaric acid were lower (−0.2 to −0.5) and often not significant. Correlations with sinapic acid were also negative and not significant.

TABLE 6. PHENOLIC ACID CONTENT OF CIMMYT POOLS (μg/g)

Pool	(*E*)-Ferulic acid	(*Z*)-Ferulic acid	*p*-Coumaric acid	Sinapic acid
25	1069a[a]	33.6abc	190. a	15.3 b
15	927ab	56.1a	136. a	27.7a
17	924ab	62.2a	196. a	16.3 b
19	903ab	45.2abc	219. a	8.9 b
26	861abc	37.2abc	75.7a	11.0 b
20	760abc	38.2abc	92.8a	8.9 b
22	673abc	40.0abc	97.5a	8.9 b
27	656abc	50.6abc	87.1a	8.5 b
18	612abc	49.8abc	76.8a	8.4 b
21	601abc	44.4abc	110. a	9.7 b
23	596abc	58.2a	90.7a	8.5 b
28	502abc	52.4abc	132. a	14.2 b
16	437 bc	54.3ab	40.2a	8.7 b
24	382 bc	23.3 bc	213. a	11.6 b
09	264 c	22.3 c	29.3a	8.5 b

[a] Means followed by the same letter are not significantly different ($P = 0.05$) in Duncan's multiple-range test. Values are a mean of four samples.

TABLE 7. CORRELATIONS BETWEEN SUSCEPTIBILITY PARAMETERS AND CHEMICAL CHARACTERISTICS IN CIMMYT POOLS

Grain phenolics	Susceptibility parameters			
	Egg number	Progeny	Consumption	Dobie index
(*E*)-Ferulic	−0.80***[a]	−0.76***	−0.76***	−0.81***
(*Z*)-Ferulic	−0.20	−0.42*	−0.50**	−0.39
p-Coumaric	−0.35	−0.37	−0.33	−0.39
Sinapic	−0.32	−0.16	−0.15	−0.35

[a] ***$P \leqslant 0.01$; **$P \leqslant 0.05$; and *$P \leqslant 0.1$. All other values $P \leqslant 0.1$.

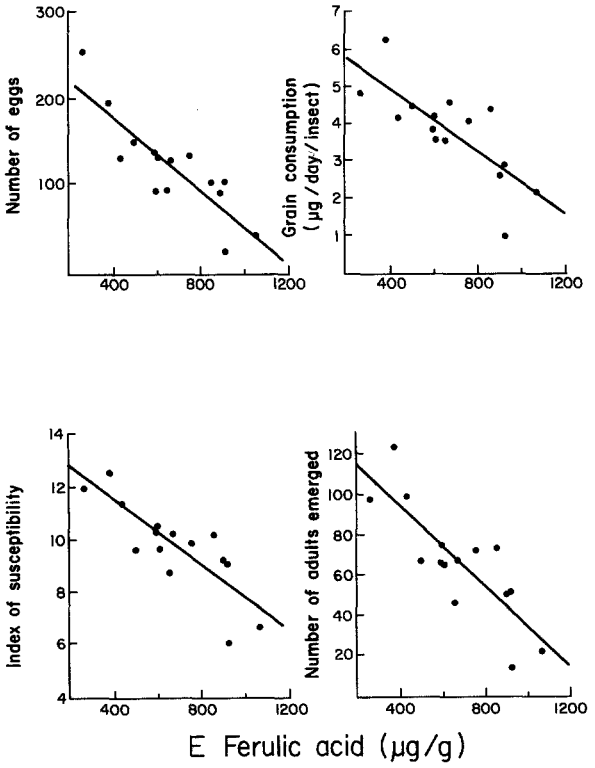

FIG. 3. Relationship between four susceptibility parameters and (*E*)-ferulic acid content of 15 CIMMYT pools.

DISCUSSION

Populations of maize with high phenolic acid content are more resistant to attack by *Sitophilus zeamais* than those with low phenolic acid content. The principal phenolic acid associated with resistance is (*E*)-ferulic acid. (*Z*)-Ferulic, *p*-coumaric, and sinapic acids may make a minor contribution. The strong negative correlation between the susceptibility parameters and the level of (*E*)-ferulic acid was observed with both land races and CIMMYT pools. Also, correlations between (*E*)-ferulic acid and susceptibility parameters were found to be of a similar magnitude as the correlations between susceptibility and the leading parameters, protein and hardness, identified in previous studies (Dobie, 1977; Eden, 1952; Singh and McCain, 1963) and confirmed in the present trials. Clearly ferulic acid can be taken as a good indicator of resistance.

Palmitic acid may play a minor role in resistance. The palmitic acid detected in the study of the land races was probably a by-product of the saponification of fats and oils and therefore reflects differing levels of these constituents. This fatty acid was significantly correlated (at $P \leq 0.05$) with only one of the susceptibility parameters, the Dobie index. Singh and McCain (1963) and Gomez et al. (1983) have reported that fat content did not affect resistance and that oil did not influence the number of eggs laid or the number of adults attracted in a free choice test. Therefore, the role of palmitic acid or fats and oils is still unclear.

In our previous study (Serratos et al., 1987) fluorescence microscopy revealed that phenolic acids are located in high concentration in the pericarp and to a lesser extent in the cell walls of the endosperm. Schoonhoven et al. (1976) have demonstrated that susceptibility increases after the removal, damage, or ether extraction of the pericarp. The importance of the pericarp, a maternally derived tissue, may also be reflected in the genetics of weevil resistance (Widstrom et al., 1975) which is known to display maternal inheritance.

At this time the mechanism by which phenolic acids contribute to resistance is unknown. While direct antifeedant effects of free ferulic acid have been demonstrated (Serratos et al., 1987), free and soluble esterified phenolic acids are a small component of the total phenolic acid content of grain. Bound phenolics form the bulk of total phenolics in maize grain (Sosulski et al., 1982), and recent [^{13}C]NMR studies (Lewis et al., 1987) have revealed that these may be linked in complex ways to carbohydrates in plant cells. It has been suggested (Fry, 1983) that bound ferulate has a strengthening effect on the structure of the cell wall and provides initiation sites for lignification. Bound ferulate is correlated with and undoubtedly contributes to seed hardness. However, further work is required on both the chemistry of phenolics in maize and their effect on stored-products insects if this interactive system is to be fully understood.

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SEX PHEROMONE OF HICKORY SHUCKWORM *Cydia caryana*
Development of an Effective Field Lure

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Abstract—Extracts of the sex pheromone glands of female *Cydia caryana* were evaluated by electroantennography and gas chromatography–mass spectrometry. These studies suggested the following compounds were potential sex pheromone components: (*Z*)-8- and/or (*E*)-9-dodecenyl acetate (50 pg/female), dodecyl acetate (40 pg/female), and (*E*, *E*)-8,10-dodecadienyl acetate (25 pg/female). In field tests only the diene produced trap catch, and when the other components were added to the diene, trap catch was not increased. When the diene was formulated in red natural rubber septa, only transient and low catches were obtained, but when gray halobutyl isoprene elastomeric septa were used, high and consistent catches were obtained for eight weeks. Catches depended on the ratio of (*E*, *E*)-8,10 to (*E*, *Z*)-8,10

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isomers. High catches were obtained for an *EE* to *EZ* ratio of 100 : 0.6, and insignificant catches were obtained when the ratio was 100 : 3. Equivalent catches were obtained for dosages of 50, 100, and 200 μg/septum.

Key Words—Sex pheromone, sex attractant, *Cydia caryana*, Lepidoptera, Tortricidae, Olethreutinae, (*Z*)-8-dodecen-1-ol acetate, (*E*)-9-dodecen-1-ol acetate, dodecanol acetate, (*E*, *E*)-8,10-dodecadien-1-ol acetate, (*E*, *Z*)-8,10-dodecadien-1-ol acetate.

INTRODUCTION

The hickory shuckworm, *Cydia caryana* (Fitch) (Lepidoptera: Tortricidae: Olethreutinae) is found throughout southeastern United States from Texas and Oklahoma in the west, to South Carolina, Georgia, and Florida in the east. *C. caryana* is one of the most destructive insect pests on pecan. Crop losses are caused by early season nut abortion and mid- to late-season hindrance of proper kernel deposition and shuck dehiscence (Payne and Heaton, 1975).

The presence of a sex pheromone in female *C. caryana* was established by Anderson (1972). Subsequently, Smith et al. (1987) determined electroantennogram (EAG) profiles of male response to monounsaturated 12- and 14-carbon alcohols and acetates and conducted studies of behavioral responses of males in a flight tunnel to the compounds that evoked the stronger EAG responses. These studies produced several candidates for use in traps.

The present study was undertaken to identify components from the female sex pheromone gland and to develop an effective field lure.

METHODS AND MATERIALS

Insects. C. caryana were obtained as full-grown overwintering larvae within pecan shucks from a pecan orchard in Sparks, Oklahoma. Larvae were maintained in a controlled environmental room, at a temperature of 22–24°C, 65% relative humidity under a 14-hr light/10-hr dark regimen. Emergent adults were collected daily, sexed, and placed in separate holding cages provided with a solution of 5% sugar-water.

Extraction of Pheromone. Female moths (two to three days after eclosion) were collected 45 min to 1 hr after the initiation of scotophase and placed in a refrigerator for 10–30 min prior to dissection. The glandular area, which lies dorsally between the 8th and 9th abdominal segments as a modified intersegmental membrane, was everted under pressure, excised, and extracted in dichloromethane at room temperture for 15 min. The solution was removed and stored at −20°C.

Gas Chromatography. The following gas chromatographic column was

used for collecting fractions of *C. caryana* female sex pheromone gland extract: a nonpolar, silanized glass column (1.8 m × 2.3 mm OD) packed with 3% dimethyl silicone (SE-30) on 80–100 mesh Gas Chrom Q and operated at 110°C for 8 min and then temperature programmed at 4°C/min to 200°C and held at that temperature.

The following capillary GC column was used for chemical analysis of biologically active fractions: a nonpolar, capillary column (60 m × 0.25 mm ID) of fused silica with cross-linked methyl silicone (DB-1) was operated at 80°C for 2 min and then temperature programmed at 20°C/min to 170°C and held at that temperature.

Collection of Sex Pheromone. Components from extract of *C. caryana* sex pheromone glands were collected from a gas chromatograph equipped with a flame ionization detector (FID) and effluent splitter. The FID to splitter ratio was 1 : 9. Effluent was collected in glass tubing traps cooled in a mixture of Dry Ice and acetone. Collection efficiency was 80%. The biological activity of collected fractions was measured by the electroantennal response of 2- to 4-day old *C. caryana* male moths.

Gas Chromatography–Mass Spectrometry (GC-MS). A quadrupole mass spectrometer (Hewlett-Packard 5970) with a GC inlet (Hewlett-Packard 5790) was used. A dimethyl silicone (DB-1) capillary column (60 m × 0.25 mm ID) was held at 80°C for 2 min and then temperature programmed at 20°C/min to 190°C and then held at that temperature.

Field Tests. Test compounds in dichloromethane were impregnated into red rubber or gray halobutyl isoprene elastomeric septa (Brown and McDonough, 1986) size 1F (The West Co., Phoenixville, Pennsylvania).

Field tests were conducted in pecan orchards in Oklahoma, Georgia, Alabama, and Kansas. Wing traps (Pherocon 1C, Trecé Corp., Salinas, California) were suspended approximately 6.2 m above ground level, within the lower to middle canopy of the pecan trees. Traps were set out in rows (replicates), with treatments assigned at random within each replicate. Traps were at least 20 m from any other trap and were separated by at least one pecan tree. Traps were checked daily; the number of *C. caryana* was recorded, and the moths were removed. Trap catch data were transformed to $(X_i + 1)^{1/2}$, and submitted to analysis of variance by Duncan's multiple-range test.

RESULTS

Identification of Sex Pheromone Gland Components. Six fractions of 94 *C. caryana* female equivalents (FE) in the volatility range of 10- through the 16-carbon acetates were collected from the packed nonpolar GC column. Electroantennograms of each fraction showed that only fractions 3 (13.5–16.5 min) and 4 (16.5–19.5 min) elicited significant responses.

Subsequent capillary GC analysis of fraction 3 showed a peak with the retention time of 12 : Ac [t_R (peak) = 14.38 min; t_R (12 : Ac) = 14.39 min]. A second peak in fraction 3 was also detected at a retention time indicative of a dodecenyl acetate (t_R = 14.26 min). Capillary GC analysis of fraction 4 indicated a peak with the same retention time as *E*8, *E*10–12 : Ac [T_R (peak) = 15.94 min; t_R (*E*8, *E*10–12 : Ac) = 15.94 min].

GC-MS analysis (selective ion monitor mode) of 18 FE of *C. caryana* whole extract gave evidence of: a dodecenyl acetate at t_R = 15.98 min and m/z = 166(M^+ − 60) and 61($CH_3CO_2H_2$), with retention time coincident with *Z*8–12 : Ac and/or *E*9–12 : Ac; 12 : Ac at t_R = 16.16 min and m/z = 168(M^+ − 60) and 61($CH_3CO_2H_2$); and a dodecadienyl acetate at t_R = 17.46 min and m/z = 224(M^+) and 164(M^+ − 60). The t_R of *E*8,*E*10–12 : Ac was also 17.46.

Quantitative evaluation of peak areas indicated that dodecenyl acetate, dodecyl acetate, and dodecadienyl acetate were present at 1.0, 0.75, and 0.5 ng in the extract of 18 female glands.

Field Tests. When trap catches provided by *E*8, *E*10–12 : Ac, *Z*8–12 : Ac, and mixtures of these compounds in gray septa were determined (Table 1, test 1), *E*8,*E*10–12 : Ac alone produced significant trap catch while *Z*8–12 : Ac did not and mixtures were less effective than *E*8,*E*10–12 : Ac alone. In test 2 (Table 1), trace quantities of *Z*8–12 : Ac(0.03–3% of *E*8,*E*10–12 : Ac) were tested. Again no lure was more effective than the one containing *E*8,*E*10–12 : Ac alone. *E*9–12 : Ac (Table 1, test 3) neither increased nor decreased trap catch provided by *E*8,*E*10–12 : Ac alone. 12 : Ac (Table 1, test 4) appeared to provide a small (44% greater) improvement in catch over *E*8,*E*10–12 : Ac alone, but the difference was not statistically significant. Dosages of *E*8,*E*10–12 : Ac of 50, 100, and 200 μg produced equivalent catches (Table 1, test 4).

When *E*8,*E*10–12 : Ac was tested in red rubber septa after aging for 0, 1, or 2 weeks, the fresh septa produced borderline catch and the older septa were ineffective (Table 2). Cotesting of the same quantity of *E*8,*E*10–12 : Ac in gray septa produced significant catch for lures aged from 0–8 weeks (Table 2).

The effect of *E*8,*Z*10–12 : Ac on efficiency of *E*8,*E*10–12 : Ac is shown in Table 3. Trap catch was high when the *EZ*% was ca. 0.6% but 3% *EZ* and higher eliminated significant catch.

DISCUSSION

Although there was not enough natural pheromone available to allow rigorous chemical proof of identity of the diene, there can be little doubt that *E*8,*E*10–12 : Ac is the principal sex pheromone component for this species.

TABLE 1. TOTAL CATCH OF MALE *Cydia caryana* (FITCH) IN FIELD TESTS[a]

Lure	Test 1 μg/dispenser	Test 1 Males/trap (21 days)	Test 2 μg/dispenser	Test 2 Males/trap (44 days)
*E*8,*E*10-12:Ac	50	290a	50	422a
*E*8,*E*10-12:Ac + Z8-12:Ac	47.5:2.5	101b	50:0.015	135b
*E*8,*E*10-12:Ac + Z8-12:Ac	35:15	33c	50:0.05	370a
*E*8,*E*10-12:Ac + Z8-12:Ac	15:35	31c	50:0.15	307a
*E*8,*E*10-12:Ac + Z8-12:Ac	2.5:47.5	48c	50:0.5	126b
*E*8,*E*10-12:Ac + Z8-12:Ac	0:50	59c	50:1.5	159b
*E*8,*E*10-12:Ac + Z8-12:Ac	0:0	24c	0:	37c
	Test 3		Test 4	
	μg/dispenser	Males/trap (45 days)	μg/dispenser	Males/trap (24 days)
*E*8,*E*10-12:Ac	50	416a		
*E*8,*E*10-12:Ac + *E*9-12:Ac	50:1.5	363a		
*E*8,*E*10-12:Ac + *E*9-12:Ac	50:5	462a		
*E*8,*E*10-12:Ac + *E*9-12:Ac	50:15	368a		
*E*8,*E*10-12:Ac + *E*9-12:Ac	0:50	12b		
*E*8,*E*10-12:Ac + *E*9-12:Ac	0:0	14b		
*E*8,*E*10-12:Ac			200	262a
*E*8,*E*10-12:Ac			100	238a
*E*8,*E*10-12:Ac			50	232a
*E*8,*E*10-12:Ac + 12:Ac			50:5	382a
*E*8,*E*10-12:Ac + 12:Ac			50:15	297a
*E*8,*E*10-12:Ac + 12:Ac			50:50	361a
*E*8,*E*10-12:Ac + 12:Ac			0:0	2b

[a]Gray elastomeric septa were used as the controlled release substrate. Tests were conducted in 1986, and there were five replicates per test. Letters after the numbers indicate significance according to Duncan's multiple range test ($P = 0.05$).

The flight tunnel and EAG studies (Smith et al., 1987), the present GC-MS and field trapping studies, and taxonomy collectively confirm this conclusion. The sex pheromones of three other *Cydia* species have been identified, and all three contained the (*E*,*E*)-8,10-dodecadienyl structure [alcohol: *C. pomonella* (Roelofs et al., 1971), and acetate: *C. latiferreanus* (Davis et al., 1984) and *C. nigricana* (Greenway, 1984)].

Although dodecyl acetate, when added to the diene did not produce sig-

TABLE 2. COMPARISON OF TRAP CATCHES (5 REPLICATES/TREATMENT) WITH 50 μg/SEPTUM OF *E*8,*E*10–12 : Ac IN RED AND GRAY SEPTA AFTER PREAGING IN THE FIELD FOR INDICATED NUMBER OF WEEKS[a]

Preliminary exposure period (weeks)	Mean catch/replicate for 2-week period	
	Red septa	Gray septa
Blanks	0.2d	0.4d
0	20.6cd	87.0bc
1	9.4d	121.2ab
2	3.6d	103.4ab
4		114.4ab
6		170.6a
7		53.0bc
8		108.8ab

[a]The tests were conducted in 1987. Letters after the numbers indicate significance according to Duncan's multiple-range test ($P = 0.05$).

nificantly increased trap catch over the diene alone, it cannot necessarily be ruled out as a possible sex pheromone component. In what may be an analogous situation, dodecanol was found to be a constituent of the sex pheromone gland extract of *C. pomonella* (Einhorn et al., 1984) and generally would not increase trap catch of *E*8,*E*10–12 : OH, but was behaviorally active at certain ratios and dosages (Arn et al., 1985).

The dodecenyl acetate, which was either *Z*8 or *E*9, did not increase trap catch when added to the diene, although it apparently accounted for the EAG activity of GC fraction 3. *Z*8–12 : Ac in combination with *E*8, *E*10–12 : Ac

TABLE 3. EFFECT OF RATIO OF *E*8,*E*10– TO *E*8,*Z*10–12 : Ac ON TRAP CATCH (10 REPLICATES/TREATMENT FOR 31 DAYS IN GRAY SEPTA)[a]

Ratio of *EE* to *EZ* (μg/septum)	Mean total catch/replicate
50 : 0.3	184.9a
50 : 1.5	11.0b
50 : 5.0	6.2b
50 : 15.0	3.2b
0 : 0	0.6b

[a]The tests were conducted in 1987. Letters after the numbers indicate significance according to Duncan's multiple-range test ($P = 0.05$).

is the sex pheromone of another Olethreutinae, *Hedya nubiferana* (Frerot et al., 1979). The reason for its presence in *C. caryana* in such large amount coupled with its inactivity in field tests is not known. Perhaps the dodecenyl acetate has the *E*9 structure and is a biosynthetic precursor to the diene. In an analogous situation *E*9–12 : OH was shown to be the biosynthetic precursor of *E*8,*E*10–12 : OH in *C. pomonella* (Löfstedt and Bengtsson, 1988).

In red rubber septa *E*8,*E*10–12 : Ac is ineffective as a lure to monitor *C. caryana* populations, most likely because of isomerization of the conjugated diene structure (Davis et al., 1984; Guerin et al., 1983; Hoffman et al., 1983). Gray septa allow much slower rates of isomerization (Brown and McDonough, 1986) and here provided an effective, long-lasting lure.

The (*E*,*Z*)-8,10-dodecadienyl structure may be as important in *Cydia* species as (*E*,*E*)-8,10-dodecadienyl. In *C. latiferreanus* (formerly *Melissopus latiferreanus*; Brown, 1983) *EZ* is the major component (*EZ* : *EE* is 80 : 20) and the pure *EE* isomer is not attractive (Davis et al. 1984). In *C. pomonella* sex pheromone gland extracts, the *EZ* : *EE* ratio is 5 : 95 (Einhorn et al., 1984). Roelofs et al. (1972) have shown that higher levels of *EZ* cause a moderate reduction in trap catch of *C. pomonella* males but the attractancy or nonattractancy of pure *EE* has not been reported. In *C. caryana* an *EE* : EZ ratio of 100 : 0.6 was very attractive in field tests, while a ratio of 100 : 3 was equivalent to blank traps. Thus, as with *C. pomonella*, it remains to be seen whether the pure *EE* isomer is attractive and whether a small quantity of *EZ* is required for best trap catch.

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IDENTIFICATION OF HOST-PLANT CHEMICALS STIMULATING OVIPOSITION BY SWALLOWTAIL BUTTERFLY, *Papilio protenor*

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Abstract—The ovipositional response of a Rutaceae-feeding papilionid butterfly, *Papilio protenor*, to *Citrus* host plants was evoked by the synergistic action of L-(−)-stachydrine, D-(−)-quinic acid, (−)-synephrine, and L-(−)-proline that characterize the chemical compositions of the leaves and epicarp of *Citrus* plants (*C. natsudaidai* and *C. unshiu*). The stimulatory activity of their mixture was enhanced by the addition of flavanone glycosides, naringin and hesperidin, which coexist in these plants and have previously been demonstrated to serve as oviposition stimulants. However, sugars such as sucrose, glucose, and inositols, which abound in plant tissues, exerted no effect on egg-laying by the females. On the other hand, chlorogenic acid present in the leaves of another host plant, *Fagara ailanthoides*, was found to act as an excellent synergist. However, there existed significant qualitative dissimilarities between the chemical compositions of the leaves of *C. unshiu* and *F. ailanthoides*. This strongly suggests that *P. protenor* is likely to utilize different categories of compounds as chemical cues in recognizing each plant as a host.

Key Words—Oviposition stimulant, *Papilio protenor*, Lepidoptera, Papilionidae, D-(−)-quinic acid, L-(−)-stachydrine, (−)-synephrine, L-(−)-proline, chlorogenic acid, *Citrus natsudaidai*, *C. unshiu*, *Fagara ailanthoides*.

INTRODUCTION

Some butterfly species depositing eggs directly on the plant where the larvae later grow have been reported to utilize primarily visual and/or olfactory cues to distinguish potential larval food plants among mixed vegetation (Vaidya, 1969; Rausher, 1978; Saxena and Goyal, 1978; Stanton, 1982; Feeny et al.,

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1983; Chew and Robbins, 1984). Earlier work on host selection in Pieridae and Papilionidae clearly demonstrates that plant metabolites serve in the final step of oviposition behavior as the cues permitting females to discriminate and to recognize their hosts (David and Gardiner, 1962; Nishida, 1977; Ichinosé and Honda, 1978; Saxena and Goyal, 1978; Rodman and Chew, 1980; Abe et al., 1981; Feeny et al., 1983). Plant chemicals are perceived by foretarsal chemotactile sensory hairs during drumming, which is essential for females to assess the suitability of the plant on which they landed (Ma and Schoonhoven, 1973; Ichinosé and Honda, 1978; Stanton, 1979).

Recently, vicenin-2, narirutin, hesperidin, rutin (flavonoids), adenosine, and 5-hydroxy-*N*, ω-methyltryptamine have been identified in the leaves of *Citrus unshiu* as oviposition stimulants for *Papilio xuthus*, which infests principally *Citrus* plants in nature (Ohsugi et al., 1985; Nishida et al., 1987). However, each of them had little or no effect on oviposition when tested alone, and only their mixture exhibited sufficient ovipositional activity.

Similar topics on the synergy of multiple components in egg-laying have also been dealt with in the cabbage butterfly, *Pieris rapae* (Renwick and Radke, 1983), and in the swallowtail butterfly, *Papilio protenor*, in which two flavanone glycosides, naringin and hesperidin, present in the epicarp of sour orange (*Citrus natsudaidai*), elicited ovipositional response from the females in combination with other unidentified water-soluble compounds (Honda, 1986). Furthermore, recent work by Feeny et al. (1988) has revealed that egg-laying in an umbellifer-feeding swallowtail butterfly, *Papilio polyxenes*, is stimulated by the synergistic action of luteolin 7-*O*-(6″-*O*-malonyl)-β-D-glucoside and *trans*-chlorogenic acid. In contrast, Pereyra and Bowers (1988) have shown that a single iridoid glycoside, catalpol, was sufficient to induce oviposition by the nymphalid butterfly, *Junonia coenia*, at levels similar to that of the host plant.

P. protenor is an oligophagous butterfly, which in nature feeds on several specific plants in the genera *Citrus* (main), *Fagara*, *Poncirus*, and so on. The present paper reports on oviposition stimulants for *P. protenor* newly identified from *Citrus* and *Fagara* host plants.

METHODS AND MATERIALS

Insects. Butterflies used for behavioral assays were 3- to 10-day-old gravid females derived from stock cultures or collected in the field in Kanagawa Prefecture.

Extraction and Fractionation of Active Components. The epicarp of sour orange, *C. natsudaidai* (760 g), which evokes potent ovipositional response (Honda, 1986), as do its leaves, was extracted with methanol (3 liter) at room temperature for one month. Young fresh leaves of *C. unshiu* and *Fagara ail-*

anthoides, which are the principal host plants of the population used in this study, were also extracted with methanol in a similar manner. Each methanolic extract was concentrated in vacuo below 50°C, and the residue, after being mixed with water, was extracted successively with chloroform and isobutanol. Since previous work (Honda, 1986) and preliminary experiments showed that the aqueous fraction derived from each extract was highly effective in releasing oviposition behavior, the water-soluble fraction was examined for chemical constituents, monitoring their oviposition stimulatory activities.

The aqueous fraction of *C. natsudaidai* was evaporated to dryness, and the residue was reextracted with hot methanol to remove resinous substances. The methanolic extract obtained (designated fraction IV) that provoked strong positive response was then subjected to TLC analysis and fractionated by means of preparative TLC (Merck PLC plate Silica gel 60).

Bioassay for Ovipositional Activity. Behavioral bioassays to evaluate the stimulatory action of test samples were conducted employing artificial green plastic foliage according to the method given in a previous paper (Honda, 1986). The rate of response was expressed by the ratio of the number of positive responses to that of alighting on the leaves that was accompanied with drumming. The appraisal of positive response was based on the following criteria: release of the ordinary sequential oviposition behavior, which consists of drumming on a leaf with the forelegs and subsequent abdominal curling, followed by settling of the ovipositor in contact with the underside of the leaf.

Instrumentation for Chemical Analyses. [^{13}C]NMR (CMR) spectra were measured at 67.8 MHz in D_2O, unless otherwise noted, with a JEOL JNM-GX 270 FT-NMR spectrometer by proton-decoupled operation. Chemical shifts were represented by δ unit from the downfield of tetramethylsilane as the external standard. The multiplicity of signals determined, by means of INEPT or off-resonance technique, was abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q).

Fast-atom-bombardment mass spectra (FAB-MS) were recorded on a JEOL JMS-DX 303 mass spectrometer. The target was bombarded with 6.0 kV Xe atoms using glycerine as a matrix, and positive and/or negative ions were recorded.

High-pressure liquid chromatography (HPLC) for the separation of optical isomers was carried out with a Hitachi 635S liquid chromatograph equipped with a wavelength-tunable spectrophotometer using a stainless column (4.6 mm ID × 250 mm) packed with Chiralpack WH (Daicel Chemical Industries, Ltd.). Mobile phase was 0.25 mM aq. $CuSO_4$ solution, and the column eluate was monitored at 225 nm. The system was operated at 40°C, and the flow rate was regulated at 1 ml/min.

The optical rotation was measured with a Horiba SEPA-200 polarimeter at 25°C in H_2O.

RESULTS

Oviposition Stimulants in the Epicarp of C. natsudaidai. Fraction IV, which was derived from the hydrosoluble part of the methanolic extract of the epicarp and which exhibited potent stimulatory activity for egg-laying, was separated by preparative TLC developing with methanol–acetone (60:40) into three fractions: A (R_f = 1.00–0.58), B (R_f = 0.58–0.27), and C (R_f = 0.27–0.00). The rates of ovipositional response of individual fractions assayed at the concentration of 0.2% were 0, 0.32, and 0.75, respectively. The most polar fraction C seems to contain some compounds of substantial importance responsible for egg-laying.

Fraction C was further fractionated by preparative TLC developed with methanol into four fractions: CA (0.68–0.45), CB (0.45–0.25), CC (0.25–0.05), and CD (0.05–0.00). The ovipositional responses of these fractions tested at a concentration of 0.5% were 0.13, 0.67, 0.17, and 0, respectively. The ovipositional activity of each fraction apparently diminished; however, the recombined sample of individual fractions resumed activity almost the same as that of the original fraction (C). This indicates that oviposition is elicited by a synergy of multiple compounds.

Since fraction CD seemed to be composed mainly of resinous substances and it induced no oviposition behavior, this fraction was discarded. An examination of the CMR spectrum of fraction CA revealed that it consists of a carboxylic acid (compound 4) and inositol isomers. Similarly, fraction CB was found to contain a large amount of another carboxylic acid (compound 1) and small quantities of compound 4 and compound 3, which was a predominant component of fraction CC. From these fractions, compounds 1, 3, and 4 were isolated by repetitive TLC as white or pale yellow crystals.

On the other hand, fraction B was shown by CMR measurement to be a mixture of an aromatic compound (compound 2), glucose, sucrose, inositols, and compound 4, while only sucrose and glucose were the major constituents of fraction A. Fraction B was subjected to TLC on silicic acid with methanol, and compound 2 was obtained as white crystals.

Structural elucidation of the four compounds 1, 2, 3, and 4 was thus attempted on the basis of the following characteristic spectral and physicochemical information:

Compound 1. Mp: 226°C. FAB-MS: $(M + H)^+$ at *m/z* 116. CMR, δ (ppm): 23.8 (t), 29.0 (t), 46.2 (t), 61.4 (d), and 174.5 (s). $[\alpha]_D^{25}$: −85.1°. This compound, of molecular weight 115 and seeming to contain one nitrogen and five carbon atoms, one of which constitutes a carboxyl group, was determined to be L-(−)-proline. To examine whether the D-isomer of proline is coexistent in fraction CB from which L-(−)-proline was isolated, fraction CB was subjected to HPLC on Chiralpack WH, which enabled the separation of the two

optical isomers. However, no peak corresponding to D-isomer (R_t = 37.0 min) was observed. Therefore, the epicarp of *C. natsudaidai* was considered to exclusively produce L-isomer (R_t = 22.5 min).

Compound 2. Mp: 169°C. FAB-MS: $(M + H)^+$ at *m/z* 168. CMR (0.1 N HCl/D_2O), δ (ppm): 33.2 (q), 54.6 (t), 68.3 (d), 115.8 (d), 127.7 (d), 131.3 (s), and 155.8 (s). $[\alpha]_D^{25}$: −50.5°. The mol wt of this substance should be 167, which is indicative of the presence of nitrogen atom(s) in its molecule. Four signals (six carbons) appearing between 110 ppm and 160 ppm represent an aromatic ring, and the resonance at 33.2 ppm appears to be a methyl group attached to the nitrogen atom. From these data compound 2 was identified as (−)-synephrine, which had already been reported in *Citrus* plants (Stewart et al., 1964; Namba et al., 1985).

Compound 3. Mp: 279°C. FAB-MS: $(M + H)^+$ at *m/z* 144. CMR, δ (ppm): 18.9 (t), 25.4 (t), 46.4 (q), 52.5 (q), 67.4 (t), 76.7 (d), and 171.0 (s). $[\alpha]_D^{25}$: −38.4°. This compound has a mol wt of 143, which is larger by just two methyl equivalents than that of proline, and it exhibits two additional resonances at 46.4 ppm and 52.5 ppm, which may be assignable to *N*-methyl carbons. These data strongly suggest that the compound is *N,N*-dimethyl betaine of proline. On the analogy of the data of L-(−)-proline, compound 3 was considered to be L-(−)-stachydrine, and the identity was confirmed by comparison with an authentic sample (Mp: 287°C, $[\alpha]_D^{25}$: −42.8°) prepared by the reaction of L-(−)-proline with two equivalents of methyl iodide in the presence of potassium carbonate in methanol.

Compound 4. Mp: 167°C. FAB-MS: $(M - H)^-$ at *m/z* 191. CMR, δ (ppm): 37.4 (t), 40.6 (t), 67.1 (d), 70.4 (d), 75.2 (d), 76.9 (s), and 180.9 (s). $[\alpha]_D^{25}$: −45.1°. This compound, of mol wt 192, possesses two methylene carbons, four hydroxyl-substituted carbons, and one carboxyl carbon, which is suggestive of a cyclic compound. Taking account of the optical rotation, compound 4 was identified as D-(−)-quinic acid.

The oviposition-stimulatory activity of the identified compounds to the females was examined next. Since sugars such as sucrose, glucose, and inositol are ubiquitous in plants, compounds 1, 2, 3, and 4 were tested first. As shown in Table 1, each substance was entirely ineffective when bioassayed alone. Furthermore, no binary mixtures were observed to display sufficient activity to release egg-laying by the females. However, the ovipositional response was markedly enhanced when an equivalent mixture of the four compounds was tested, although the stimulatory activity of this quaternary system did not reach the level of fraction IV.

Another experiment was carried out to estimate the degree of contribution to the oviposition of individual compounds and two flavonoid compounds, naringin and hesperidin, both of which have already been reported to act as synergists in the oviposition of *P. protenor* (Honda, 1986). Because a sufficient

TABLE 1. OVIPOSITIONAL RESPONSE OF *Papilio protenor* TO CHEMICALS CONTAINED IN *Citrus* HOST PLANTS

Sample	Response (No. of trials)[a]		
	0.2%[b]	0.5%	1.0%
Fraction IV	0.92 (25)	1.00 (25)	1.00 (22)
L-(−)-Proline (1)			0 (25)
(−)-Synephrine (2)			0 (25)
L-(−)-Stachydrine (3)			0 (25)
D-(−)-Quinic acid (4)			0 (24)
1 + 2 + 3 + 4[c]	0.22 (46)	0.61 (46)	0.71 (45)

[a] Fourteen females were tested.
[b] Percent indicates concentration of sample.
[c] Equivalent mixture of each compound.

quantity of optically active natural (−)-synephrine was not available, commercial D,L-synephrine was used in this test. The results are given in Table 2.

The remarkable synergistic action of naringin and/or hesperidin on oviposition was reconfirmed through this experiment. At first the author underestimated the function of flavonoids in egg-laying; however, they proved of significant influence. The effectiveness of quinic acid and stachydrine was also prominent, whereas that of synephrine was moderate, and proline was found to

TABLE 2. EFFECT OF *Citrus* PLANT CHEMICALS ON OVIPOSITION BY *Papilio protenor*

Compound (%)	Sample[a]						
	A	B	C	D	E	F	G
L-(−)-Proline (0.2)	*		*		*	*	*
D,L-Synephrine (0.1)	*		*	*		*	*
L-(−)-Stachydrine (0.2)	*		*	*	*		*
D-(−)-Quinic acid (0.2)	*		*	*	*	*	
Naringin (0.1)		*	*	*	*	*	*
Hesperidin (0.05)		*	*	*	*	*	*
Response[b]	0.64	0	0.88	0.80	0.70	0.38	0.27
No. of trials[c]	86	45	92	92	92	94	90

[a] In each sample asterisk denotes the compound added.
[b] In comparison with sample C, the response of every sample except D was significantly different at $P = 0.01$ (χ^2 test).
[c] Eighteen females were tested.

be much less responsible for oviposition. The comparatively weaker activity of synephrine may be due to the coexistence of a (+)-isomer, which is deemed to be absent in the original plant. This problem should be resolved by further investigation. At any rate, it is evident that all of these compounds, including flavanones, concurrently display significant stimulatory activity for egg-laying by *P. protenor*.

Oviposition Stimulants in Leaves of C. unshiu. Although these substances derived from the epicarp of *C. natsudaidai* synergistically induce oviposition by the females, it is not yet elucidated if they are the real oviposition releasers present in the leaves of the host plant, for the females in nature ordinarily deposit eggs not on fruit but exclusively on leaves and, sometimes, on twigs. To elucidate this question, analysis of the chemical composition of young leaves of *C. unshiu* was conducted of the water-soluble fraction. As shown in Figure 1, the compounds found in the epicarp of *C. natsudaidai* were also detected from the leaves of *C. unshiu* and in much higher concentration. Despite the great difference between the two in the absolute concentrations of the individual components, the overall profile of the relative proportion of each constituent in the leaves was quite similar to that of the epicarp, except for L-(−)-proline, which exhibited weak ovipositional activity. Therefore, it was definitely substantiated that compounds 1 to 4 are the actual oviposition stimulants in the leaves of *C. unshiu*, although other unidentified compounds would also play an important role in host discrimination and preference.

Oviposition Stimulants in Leaves of Fagara ailanthoides. *P. protenor* occa-

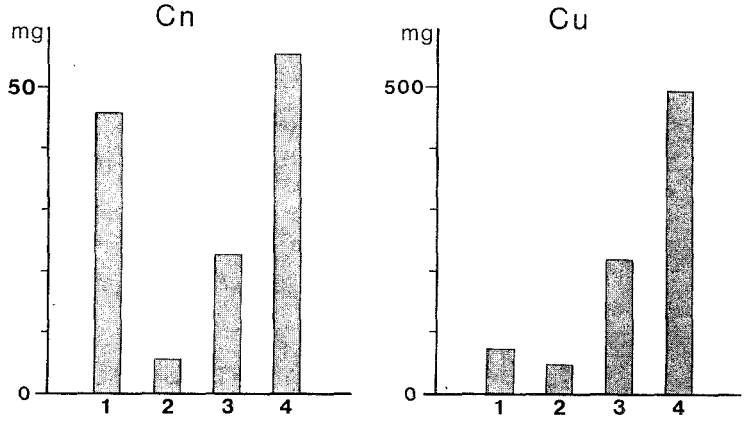

FIG. 1. Concentrations of some characteristic compounds in *Citrus* plants (per 100 g of fresh weight) that stimulate oviposition by *Papilio protenor*. Cn: *C. natsudaidai* (epicarp), Cu: *C. unshiu* (leaf). 1: L-(−)-proline, 2: (−)-synephrine, 3: L-(−)-stachydrine, 4: D-(−)-quinic acid.

sionally utilizes *F. ailanthoides* as a host in a habitat abundant in this plant. By analogous extraction and separation procedures to those presented for the epicarp of *C. natsudaidai*, another substance (compound 5) showing the following spectral characteristics was obtained as crude crystals. FAB-MS: $(M + H)^+$ at *m/z* 355. CMR (0.1 N KOH/D_2O), δ (ppm): 37.0 (t), 38.0 (t), 70.3 (d), 70.9 (d), 72.5 (d), 76.3 (s), 114.1 (d), 115.0 (d), 116.0 (d), 122.6 (d), 126.8 (s), 144.0 (s), 146.0 (d), 146.9 (s), 168.8 (s), and 179.8 (s). Although purification of this compound was unsuccessful, compound 5 was concluded to be chlorogenic acid by comparison of its spectral data with those of an authentic compound. The concentration of this compound in young leaves of *F. ailanthoides* was estimated at ca. 230 mg/100 g of fresh weight.

Further investigation on the chemical constituents in the leaves of *C. unshiu* and *F. ailanthoides* revealed the existence of large amounts of sugars, such as sucrose, glucose, and inositols. Thereupon, the effect of these sugars and chlorogenic acid on the ovipositional response of *P. protenor* was examined in combination with the oviposition stimulants so far identified. In this experiment, females that made little or no response to sample C (Table 3), but which responded positively to the leaves of *C. unshiu*, were chosen selectively to

TABLE 3. EFFECT OF SUGARS AND CHLOROGENIC ACID ON OVIPOSITION BY *Papilio protenor*

	Sample[a]						
Compound (%)	A	H	I	C	J	K	L
L-(−)-Proline (0.2)	*	*	*	*	*	*	
D,L-Synephrine (0.1)	*	*	*	*	*	*	
L-(−)-Stachydrine (0.2)	*	*	*	*	*	*	
D-(−)-Quinic acid (0.2)	*	*	*	*	*	*	
Naringin (0.1)				*	*	*	
Hesperidin (0.05)				*	*	*	
Sucrose (0.5)		*			*		*
Glucose (0.5)		*			*		*
Inositols (0.5)[b]		*			*		*
Chlorogenic acid (0.1)			*			*	*
Response[c]	0.13x	0.09x	0.39y	0.16x	0.17x	0.63y	0.09
No. of trials[d]	24	23	23	25	24	27	22

[a] In each sample asterisk denotes the compound added.
[b] Mixture of stereoisomers of inositol comprizing myoinositol (major) and chiroinositol.
[c] In each group of sample, values followed by the same letter are not significantly different at $P = 0.05$ (χ^2 test).
[d] Eight females that made little or no response to sample C were used.

appraise the influence of these compounds. Such females were found in a proportion of 10–20% in the population studied. They are not anomalies, and individual variation in oviposition preference in which chemical stimuli appear profoundly involved is quite commonplace in many butterflies (Tabashnik et al., 1981; Wiklund, 1981; Moore, 1986; Thompson, 1988). The results are summarized in Table 3. It is apparent that a sugar mixture composed of sucrose, glucose, and inositols exerts neither a stimulatory nor a synergistic effect on egg-laying (samples H and J), despite their abundance in the host plants. The combination of sugars and chlorogenic acid also did not evoke any ovipositional response by themselves (sample L), and inactivity of these compounds was further corroborated by carrying out an assay with normal females that responded positively to both samples A and C. Unlike sugars, chlorogenic acid notably enhanced the activity for egg-laying when admixed with sample A or C (sample I or K), which undoubtedly demonstrates that chlorogenic acid is also an oviposition stimulant acting synergistically. Moreover, the rate of ovipositional response to sample K was found to run up to approximately 1.0 ($N = 14$) when bioassayed with normal females.

Besides these compounds, two other compounds, neohesperidin and caffeic acid, which are related to hesperidin and chlorogenic acid, respectively, were tested for their ovipositional activity. Neohesperidin has been reported to be one of major flavanone glycosides present in the leaves and peel of *C. natsudaidai* (Nishiura et al., 1969; Tosa et al., 1988), and caffeic acid corresponds to the aglycone of chlorogenic acid, although the presence of caffeic acid in *F. ailanthoides* has not yet been ascertained. Neither of these compounds, however, affected oviposition behavior at concentrations of 0.05 or 0.1%. Consequently, seven compounds in total, including two flavanone glycosides, were reckoned as active for egg-laying by the females of *P. protenor*. The chemical structures of oviposition stimulants newly identified in this study are shown in Figure 2.

DISCUSSION

These results, combined with previous work (Honda, 1986), substantiate that host recognition in *P. protenor* is mediated by a variety of secondary plant metabolites in wide categories of chemical compounds such as sugar acid, amino acid, alkaloid, flavonoid, and so on. It is interesting that plant metabolites that would be of little nutritional importance to larvae are dominant determinants of host selection, whereas sugars, which are essential nutrients, do not exert a synergistic effect on oviposition at all. This appears true with other systems of host selection in the Pieridae (David and Gardiner, 1962), Papilionidae (Ohsugi et al., 1985; Nishida et al., 1987; Feeny et al., 1988), and Nymphalidae (Per-

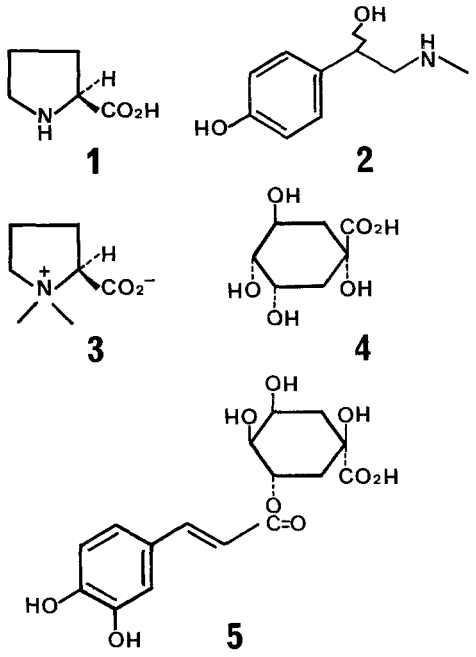

FIG. 2. Chemical structures of oviposition stimulants for *Papilio protenor* newly identified from its host plants. 1: L-(−)-proline, 2: (−)-synephrine, 3: L-(−)-stachydrine, 4: D-(−)-quinic acid, 5: chlorogenic acid. Chlorogenic acid was found in *Fagara ailanthoides*, and other compounds, in *Citrus unshiu* and *C. natsudaidai*.

eyra and Bowers, 1988). Taking into account the fact that the nutritional profiles of most plants are more or less the same, the history of host selection in butterflies might be the evolutionary process of defensive tactics focused on avoidance or detoxification of harmful plant allelochemicals, rather than a process of positive exploitation of a new host range.

Among the compounds presently appraised as active in oviposition behavior, synephrine, to the best of our knowledge, is specific to Rutaceae, and *Citrus* plants are excellent producers (Stewart et al., 1964; Namba et al., 1985). On the other hand, stachydrine occurs also in several genera other than *Citrus*, and quinic acid and proline are ubiquitous in the plant kingdom. Even so, these substances, which, by quantitative examination, proved to be major components in the water-soluble fraction of the leaves of *C. unshiu*, characterize the chemistry of the plant. Upon further investigation, the leaves of *C. natsudaidai* also contained all these compounds in somewhat different proportions, thereby suggesting that they are specific oviposition stimulants for *P. protenor*, which certain *Citrus* plants have in common. In this respect, it is highly likely that host recognition in *P. protenor* is governed by both series of compounds,

including two flavanones, that are qualitatively specific to and quantitatively characteristic of *Citrus* plants. Similar instances implicating multiple components for host discrimination or host preference have been demonstrated for *Papilio xuthus* (Nishida et al., 1987), *Papilio polyxenes* (Feeny et al., 1988), and *Pieris rapae* (Renwick and Radke, 1983, 1987, 1988). By contrast, in a nymphalid butterfly, *Junonia coenia*, a single iridoid glycoside (catalpol) suffices to elicit oviposition (Pereyra and Bowers, 1988).

Since chlorogenic acid found in *F. ailanthoides* markedly enhanced ovipositional response when added to the mixture based on *Citrus* plant materials, the acid can be regarded as a potent synergistic stimulant. The synergy of chlorogenic acid in oviposition is also known in *P. polyxenes* (Feeny et al., 1988). Although chlorogenic acid displayed striking stimulatory activity, there was no evidence of its existence in *Citrus* plants. Therefore, it is questionable whether chlorogenic acid is a real key substance acting concurrently with *Citrus* chemicals in nature. In addition, a brief chemical analysis revealed that the chemical compositions of *C. unshiu* and *F. ailanthoides* were quite different in quality from each other. In fact, young leaves of *F. ailanthoides* contained no appreciable amounts of quinic acid, stachydrine, synephrine, hesperidin, and naringin, and, except for sugar components, chlorogenic acid was one of principal constituents of this plant. These facts provide a piece of evidence that egg-laying on *F. ailanthoides* is triggered by a distinct group of compounds that include chlorogenic acid from the active compounds identified in *Citrus* plants.

P. protenor and *P. xuthus* are sympatric in most localities, and either species intrinsically depends on *Citrus* plants for larval feeding. However, the compounds with ovipositional activity to *P. protenor* are somewhat different from those reported for *P. xuthus* (Nishida et al., 1987), notwithstanding that the plant species from which oviposition stimulants were identified was actually the same (*C. unshiu*). Among the compounds shown to serve as oviposition stimulants for *P. xuthus*, 5-hydroxy-*N*,ω-methyltryptamine and adenosine were found entirely inactive to *P. protenor* (Honda, unpublished). While *P. protenor* shows positive response only to certain specified flavanone glycosides (Honda, 1986), diverse flavonoid compounds are stimulatory to *P. xuthus* (Nishida et al., 1987). It is very intriguing that host discrimination in these related butterflies utilizing virtually the same plant as a host is mediated by different groups of plant chemicals. This finding seems to give us new insight into chemical factors as a major element underlying the evolution of host selection and coevolution with plants in butterflies.

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NATURAL PRODUCTS PHYTOTOXICITY
A Bioassay Suitable for Small Quantities of Slightly Water-Soluble Compounds[1]

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Abstract—A large variety of secondary metabolites that can inhibit germination and/or seedling growth are produced by plants in low quantities. The objective of this study was to develop a bioassay capable of reliably assessing reductions in germination percentage and seedling length of small-seeded plant species caused by exposure to minute quantities of these compounds. The germination and growth of alfalfa (*Medicago sativa*), annual ryegrass (*Lolium multiflorum*), and velvetleaf (*Abutilon theophrasti*) were evaluated against six known phytotoxins from five chemical classes; cinmethylin (a herbicidal cineole derivative) was selected as a comparison standard. Each phytotoxin, dissolved in a suitable organic solvent, was placed on water–agar in small tissue culture wells. After the solvent evaporated, imbibed seeds were placed on the agar; after three days, germination percentages and seedling lengths were measured. Compared to a commonly used filter paper procedure, this modified agar bioassay required smaller quantities of compound per seed for comparable bioassay results. This bioassay also readily permitted the measurement of seedling length, a more sensitive indicator of phytotoxicity than germination. Seedling length decreased sigmoidally as the toxin concentration increased logarithmically. Phytotoxicity was a function of both compound and plant species. Cinmethylin, a grass herbicide, reduced the length of annual ryegrass seedlings by 90–100%, whereas that of alfalfa and velvetleaf was inhibited slightly. The agar bioassay facilitated the rapid and reliable testing of slightly water-soluble compounds, requiring only minute quantities of each compound to give reproducible results.

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Key Words—Alfalfa, *Medicago sativa*, benzylisothiocyanate, cinmethylin, cinnamaldehyde, coumarin, germination, juglone, nigericin, plumbagin, ryegrass, *Lolium multiflorum*, seedling length, velvetleaf, *Abutilon theophrasti*.

INTRODUCTION

The phytotoxicity of various compounds, as demonstrated by reductions in germination and seedling growth, has been evaluated in Petri dishes with filter paper (Lehle and Putnam, 1982; Shilling and Yoshikawa, 1987; Tang and Young, 1982; Wolf, 1986), sand (El-Deek and Hess, 1986), and water agar (Pederson, 1986). Although the germination percentage (defined as the proportion of seeds with a protruded radicle) is typically measured to assess the phytotoxic response to a compound, root length and root fresh weight were found to be more sensitive growth parameters (Cope, 1982; Pederson, 1986; Shilling and Yoshikawa, 1987). Because bubbles form under filter paper when wetted, small seeds often received a nonuniform supply of moisture and gave inconsistent results. Pederson (1986) found that differences in white clover (*Trifolium repens*) root length were more readily detected when grown on agar treated with aqueous extracts from tall fescue (*Festuca arundinacea*) leaves than on germination paper.

The majority of phytotoxicity studies have been conducted with water-soluble compounds such as the phenolics. These materials are typically applied to a growth medium in aqueous solution (Leather and Einhellig, 1985; Lehle and Putnam, 1982; Tang and Young, 1982). Pederson (1986) combined liquid agar and aqueous leaf extracts and then allowed them to solidify prior to assay. Other phytotoxic compounds, such as nigericin, are poorly soluble in aqueous systems (Heisey and Putnam, 1986). These toxins are normally dissolved in organic solvents and applied to filter paper. After the solvent is evaporated, water is added to induce germination (Shilling and Yoshikawa, 1987; Wolf et al., 1984; Wolf, 1986). The application of slightly water-soluble compounds to an agar surface may facilitate the accurate measurement of their toxic effects on the seedling length of small-seeded plant species.

The application of phytotoxins to a relatively large surface area, such as 100-mm-diameter Petri dishes (Wolf et al., 1984; Pederson, 1986), requires substantial quantities of test materials. By reducing the area of the test surface, a greater proportion of the slightly water-soluble compound is in contact with the seedling. The quantity of material available for study is often limited in natural products research, precluding the accurate bioassay of toxic effects. A reduction in the dimensions of the germination container might reduce the quantity of compound required for replicated bioassay. The objective of this study was to develop a bioassay capable of reliably assessing effects of minimum

quantities of phytotoxin on germination and seedling growth of several small-seeded plant species. The toxicity of six phytotoxins from five chemical classes, and the grass herbicide cinmethylin, was evaluated on three plant species with an agar germination medium in small tissue culture wells.

METHODS AND MATERIALS

Source of Materials. Alfalfa (*Medicago sativa* L., cv. Vernal, Kelly Seed Co., Peoria, Illinois), annual ryegrass (*Lolium multiflorum* L., Kelly Seed Co.), and velvetleaf (*Abutilon theophrasti* Medic., Azlin Weed Seed Service, Leland, Mississippi) seeds were obtained from cold storage (1°C) at the Northern Regional Research Center (NRRC), Peoria, Illinois). Cinmethylin {*exo*-1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-7-oxabicyclo[2.2.1] heptane} was supplied by E. I. du Pont de Nemours and Co., Inc., Wilmington, Delaware. *trans*-Cinnamaldehyde, coumarin, and nigericin were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin; juglone and plumbagin from Sigma Chemical Company, St. Louis, Missouri; and benzylisothiocyanate from ICN Pharmaceuticals, Inc., Plainview, New York. Cluster24 tissue culture dishes were obtained from Costar, Cambridge, Massachusetts. All solvents were reagent grade.

Solvent Optimization. Water agar was prepared by autoclaving 4.4 g Bactoagar (Difco Laboratory, Detroit, Michigan) in 400 ml distilled water for 20 min, and then diluted with an additional 400 ml of distilled water. One milliliter of agar was placed in each of the 24 3-ml wells, arranged in four rows of six columns each, in each disposable tissue culture dish. The volumes 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 ml of four solvent mixtures (100:0, 10:90, 1:99, or 0:100 of chloroform–hexane) were layered on solidified agar in each tissue culture well. The solvent was allowed to passively evaporate from the agar surface in a ventilated hood, typically requiring 4 hr, before seeds were placed on the agar.

Alfalfa and velvetleaf seeds were surface-sterilized in 10% Clorox for 15 m, thoroughly rinsed with distilled water, and imbibed for 4 hr on two layers of filter paper that were saturated with water. Five seeds of imbibed alfalfa and velvetleaf, and of unimbibed annual ryegrass, were placed on the agar in each well. The dishes were placed in a growth chamber at constant 25°C. After three days, seeds with a radicle protruding through the seed coat were counted as germinated. The seedling length (root plus shoot) of germinated seeds was measured after being frozen. Freezing over night softened the seedlings and facilitated the measurement of length. Seeds that did not germinate were not considered in seedling length measurements.

Agar Bioassay Method. A stock solution of each test compound was made

by dissolving the desired quantity in 1 ml chloroform and diluting to 100 ml with hexane. A dosage series of each compound was made by diluting the stock solution with 1:99 chloroform–hexane (v/v). One milliliter of each solution was carefully layered on the agar in each appropriate tissue culture well. Five imbibed alfalfa, annual ryegrass, or velvetleaf seeds were placed in each well. Germination percentages and seedling lengths were recorded according to the procedure described above.

Filter Paper Bioassay Method. Filter paper germination bioassays were conducted as described by Wolf et al. (1984). Two pieces of Whatman No. 1 filter paper were placed in each 9.0-cm-diameter glass Petri dish. Two milliliters of each compound, dissolved in 1:99 chloroform–hexane (v/v) as before, were pipetted on the filter paper. Approximately 1 hr after evaporating to dryness, the filter paper was rewetted with 4 ml distilled water and 20 imbibed seeds were spaced on the filter paper. The dishes were wrapped in aluminum foil and placed in a growth chamber maintained at 25°C. After three days, the number of germinated seeds was counted according to the procedure described previously.

Experimental Design. Solvent and germination bioassays were conducted as split-plot completely randomized designs. Toxin or solvent mixture represented the main plots, and the dose represented the split plot. All experiments were replicated at least four times. The data were analyzed by general linear model and linear regression procedures of the Statistical Analysis System (SAS Innstitute Inc., Cary, North Carolina 27511-8000) and the curvilinear regression procedure of SigmaPlot (Jandel Scientific, Corte Madera, California 94925). Treatment effects were considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Solvent Optimization. Many biologically active compounds isolated in natural products research are insoluble or slightly soluble in water, but are soluble in organic solvents. Because chloroform and hexane are inexpensive solvents that can readily dissolve a wide variety of poorly water-soluble compounds, an optimum solvent or solvent mixture for compound delivery was identified using these solvents. Alfalfa and velvetleaf germination and seedling length were equally affected by the solvent treatments (Figure 1). Only 0.2 ml chloroform reduced the germination percentage of alfalfa and velvetleaf by 80% (Figure 1). A volume of 0.4 ml chloroform or greater reduced the germination percentage by 100%. Seedling length was reduced 77% by 0.2 ml of chloroform, and 100% by 0.4 ml or more chloroform (Figure 1). Chloroform dissolved the surface of the polystyrene wells leaving a layer impervious to water movement between the seed and agar. Chloroform also penetrated the agar because its

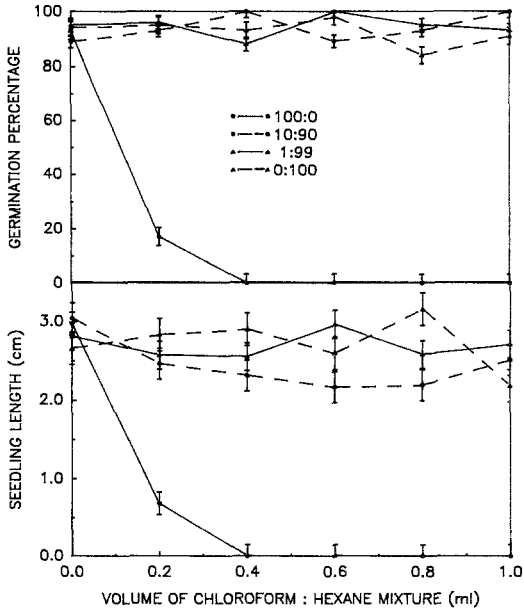

FIG. 1. The effect of chloroform, hexane, and representative mixtures of chloroform and hexane on alfalfa and velvetleaf seed germination and early seedling growth in polystyrene tissue culture wells ± 1 SE.

density is greater than that of water, preventing its complete evaporation from the agar.

Germination percentage was not affected by absolute hexane or either of the chloroform–hexane mixtures (Figure 1). Seedling length was reduced by 25% at all volumes of 10:90 chloroform–hexane, but was not reduced by hexane or 1:99 chloroform–hexane (Figure 1). Absolute hexane did not affect germination or seedling growth in the bioassay after evaporation but was incapable of dissolving several of the test compounds. A small proportion of chloroform (10:90, chloroform–hexane, v/v) proved nontoxic to germination, but reduced seedling length slightly. The optimum solvent was a 1:99 mixture of chloroform–hexane (v/v), which was nontoxic to both germination and seedling growth. When dissolved in a small volume of chloroform and diluted to the desired volume with hexane, all the test compounds, plus numerous others not reported here, could be solubilized and tested for phytotoxicity using the agar bioassay without an adverse solvent effect.

Agar versus Filter Paper Bioassay. All five compounds submitted to filter paper and agar bioassays reduced the germination of alfalfa, annual ryegrass, and velvetleaf to at least 20%, except for benzylisothiocyanate against vel-

vetleaf on filter paper (Tables 1–5). Both bioassay techniques indicated that juglone, plumbagin, and coumarin were more phytotoxic against germination than benzylisothiocyanate and cinnamaldehyde. A statistically significant curvilinear regression equation was fitted to the data for each plant–compound combination, indicating that the germination percentage was reduced linearly as the concentration of phytotoxin decreased. Using the agar bioassay, the LD_{50} (mg/replication) of juglone and plumbagin against velvetleaf was 20- and fourfold less, respectively, than the filter paper bioassay (Table 1). Similarly, between three- and sixfold less juglone or plumbagin was required to reduce the germination percentage of alfalfa or annual ryegrass with the agar bioassay (Table 2). With coumarin, the LD_{50} (mg/replication) values for alfalfa and annual ryegrass were six- and ninefold less with the agar bioassay (Table 3). Much lower doses of benzylisothiocyanate and cinnamaldehyde (26- to 187-fold) were required by the agar bioassay to reduce the germination percentages of alfalfa, annual ryegrass, and velvetleaf comparably to that of the filter paper bioassay (Tables 4 and 5). Without exception, the agar bioassay described herein

TABLE 1. RELATIVE PHYTOTOXICITY OF JUGLONE AND PLUMBAGIN AGAINST VELVETLEAF SEED GERMINATION IN AGAR AND FILTER PAPER BIOASSAY

	Filter paper bioassay[a]			Agar bioassay		
	mg/rep	Mole/seed (10^{-8})	Germination (%)	mg/rep	Mole/seed (10^{-8})	Germination (%)
Juglone						
	0.28	8	100	0	0	95
	0.56	16	74	0.017	2	80
	0.70	20	41	0.035	4	60
	2.09	60	6	0.053	6	35
				0.070	8	25
				0.088	10	10
LD_{50}	1.02	30		0.05	5	
$LSD_{0.05}$						14
Plumbagin						
	0.15	4	100	0	0	92
	0.38	10	66	0.031	3.3	92
	0.53	14	22	0.063	6.7	67
	0.60	16	12	0.094	10.0	50
				0.125	13.3	33
				0.157	16.7	17
LD_{50}	0.40	9		0.10	10	
$LSD_{0.05}$						22

[a] From Spencer et al. (1986).

TABLE 2. RELATIVE PHYTOTOXICITY OF 1,4-NAPHTHOQUINONES JUGLONE AND PLUMBAGIN AGAINST ALFALFA AND ANNUAL RYEGRASS SEED GERMINATION IN AGAR AND FILTER PAPER BIOASSAY

Filter paper bioassay				Agar bioassay			
		Germination (%)				Germination (%)	
mg/rep	Mole/seed (10^{-8})	Alfalfa	Ryegrass	mg/rep	Mole/seed (10^{-8})	Alfalfa	Ryegrass
Juglone							
0	0	95	97	0	0	95	90
0.003	0.1	92	95	0.017	2	90	95
0.035	1.0	87	97	0.052	6	80	55
0.348	10.0	58	80	0.087	10	70	45
0.523	15.0	46	33	0.131	15	20	0
0.697	20.0	20	11	0.174	20	7	0
LD_{50}							
mg/rep		0.43	0.43			0.10	0.07
Mole/seed		13.4	12.1			10.8	7.9
$LSD_{0.05}$		16	10			21	17
Plumbagin							
0	0	97	95	0	0	95	95
0.075	2.0	90	100	0.019	2	85	55
0.151	4.0	92	90	0.056	6	100	0
0.226	6.0	8	0	0.094	10	60	0
0.565	15.0	0	0	0.141	15	0	0
LD_{50}							
mg/rep		0.24	0.17			0.09	0.03
Mole/seed		4.2	3.9			9.0	2.9
$LSD_{0.05}$	9	9	4		18	18	10

TABLE 3. RELATIVE PHYTOTOXICITY OF COUMARIN IN AGAR AND FILTER PAPER BIOASSAY WITH ALFALFA, ANNUAL RYEGRASS, AND VELVETLEAF

Dose		Germination percentage		
mg/rep	Mole/seed (10^{-8})	Alfalfa	Annual ryegrass	Velvetleaf
Filter paper bioassay				
0	0	92	93	95
0.003	0.1	92	92	95
0.029	1.0	77	90	90
0.292	10.0	53	48	98
0.438	15.0	6	18	94
0.584	20.0	6	4	89
LD_{50}				
mg/rep		0.24	0.26	>0.58
Mole/seed		9.9	10.1	>20.0
$LSD_{0.05}$		18	34	8
Agar bioassay				
0	0	95	90	100
0.029	4	70	75	100
0.044	6	20	20	83
0.073	10	15	0	92
0.110	15	0	0	0
LD_{50}				
mg/rep		0.04	0.03	0.08
Mole/seed		6.6	4.9	9.6
$LSD_{0.05}$		16	13	16

required smaller quantities of the compounds tested to reduce the germination percentage comparably to that of the typical filter paper bioassay.

The agar bioassay provides a sensitive method for screening the phytotoxicity of both water- (Pederson, 1986) and organically soluble compounds that are available in limited quantity. One reason less compound was required for a similar effect is because of the smaller dimensions of the tissue culture wells in comparison to that of the Petri dishes. Poorly water-soluble compounds can be absorbed by seeds or seedlings through direct root contact or at dilute concentrations in the bulk flow of water to the root. A larger proportion of poorly water-soluble or insoluble molecules placed on the agar would be available for absorption by the seedlings because a greater proportion of the agar surface is in contact with a seedling, improving the efficiency of phytotoxin contact with, and uptake by, the seedlings.

TABLE 4. RELATIVE PHYTOTOXICITY OF BENZYLISOTHIOCYANATE IN AGAR AND FILTER PAPER BIOASSAY WITH ALFALFA, ANNUAL RYEGRASS, AND VELVETLEAF

Dose		Germination percentage		
mg/rep	Mole/seed (10^{-8})	Alfalfa	Annual ryegrass	Velvetleaf
Filter paper bioassay				
0	0	94	98	94
0.015	0.5	80	95	94
0.147	5.0	89	86	93
1.470	50.0	91	94	94
14.700	500.0	0	0	0
LD_{50}				
mg/rep		6.72	7.08	7.08
Mole/seed		252.7	251.8	251.8
$LSD_{0.05}$		10	8	6
Agar bioassay				
0	0	83	97	90
0.004	0.5	87	90	93
0.037	5.0	90	80	93
0.368	50.0	33	0	0
3.675	500.0	0	0	3
LD_{50}				
mg/rep		0.26	0.17	0.18
Mole/seed		40.3	24.9	25.2
$LSD_{0.05}$		30	12	16

A second reason for the requirement of less compound was because the efficacy of several compounds, namely benzylisothiocyanate and cinnamaldehyde, was enhanced in the agar bioassay. The LD_{50} values in mole per seed units for benzylisothiocyanate and cinnamaldehyde were approximately 10-fold less in the agar bioassay than the filter paper bioassay. The LD_{50} values (mole/seed) of juglone, plumbagin, and coumarin varied from sixfold less to no different in the agar bioassay than the filter paper bioassay. The interaction between compound and bioassay medium may be due to differences such as compound solubility and polarity.

Germination Percentage and Seedling Length. The agar bioassay can capably detect reductions in seedling length by phytotoxin doses lower than those required to detect reductions in the germination percentage, further minimizing the quantity of compound required for bioassay. After seedlings are killed and

TABLE 5. RELATIVE PHYTOTOXICITY OF *t*-CINNAMELDAHYDE IN AGAR AND FILTER PAPER BIOASSAY WITH ALFALFA, ANNUAL RYEGRASS, AND VELVETLEAF

Dose		Germination percentage		
mg/rep	Mole/seed (10^{-8})	Alfalfa	Annual ryegrass	Velvetleaf
Filter paper bioassay				
0	0	94	98	94
0.013	0.5	94	94	96
0.132	5.0	89	95	94
1.322	50.0	91	70	95
13.215	500.0	5	0	13
LD_{50}				
mg/rep		6.57	5.92	7.46
Mole/seed		265.2	246.9	290.8
$LSD_{0.05}$		17	26	26
Agar bioassay				
0	0	87	93	92
0.003	0.5	100	73	93
0.033	5.0	77	90	53
0.330	50.0	0	0	0
3.304	500.0	0	0	3
LD_{50}				
mg/rep		0.15	0.14	0.04
Mole/seed		24.9	25.2	24.4
$LSD_{0.05}$		17	26	26

softened by freezing, seedling length can be readily and reliably measured using the agar bioassay. The roots are restricted to the filter paper surface and can be difficult to remove from the paper without breaking, whereas they readily penetrate the agar and are easily removed. The germination percentage LD_{50} and the seedling length ID_{50} in the agar bioassay, when averaged across the plant species tested, were 8 and 6 $\times$ 10^{-8} mole/seed for juglone and plumbagin, and 7 and 2 $\times$ 10^{-8} mole/seed for coumarin (Tables 1–5, Figure 2), respectively. Others have also shown that many compounds can reduce seedling length (Cope, 1982; Pederson, 1986) and root length (Shilling and Yoshikawa, 1987) by a greater extent than germination at toxic concentrations.

In addition to improving the sensitivity of the bioassay, the measurement of seedling length permits identification of certain compounds as being toxic that would be overlooked if considering germination percentage alone. Rela-

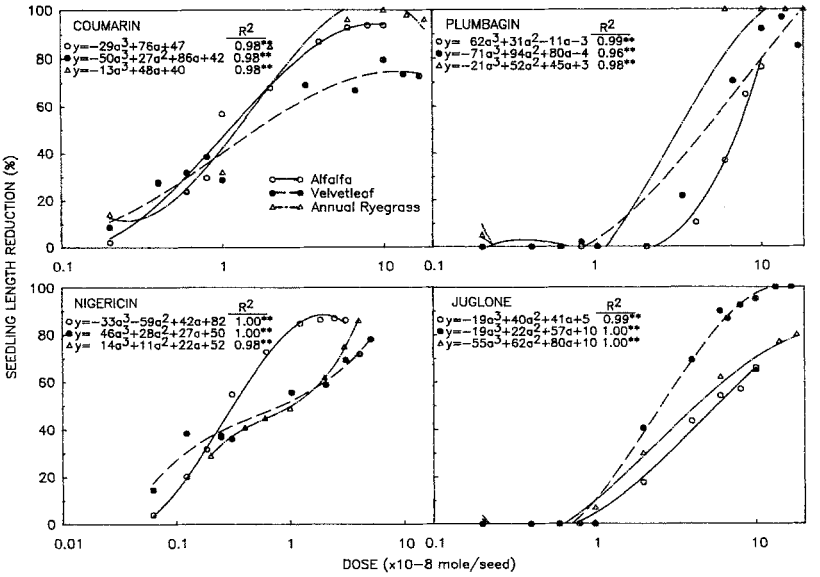

FIG. 2. Reductions in the seedling growth of alfalfa, annual ryegrass, and velvetleaf resulting from increased dosages of several phytotoxins delivered using the modified bioassay (** indicates significance at the 1% level).

tively few compounds directly inhibit the germination process. Many compounds, however, inhibit one or more aspects of seedling growth and metabolism. Nigericin did not inhibit the germination of alfalfa, annual ryegrass, or velvetleaf at the highest doses (data not shown), but was substantially more inhibitory to seedling growth than any of the other compounds tested (Figure 2). Greater potential for identifying inhibitory compounds may be realized by measuring a general parameter of seedling growth such as length.

A sigmoidal reduction in alfalfa, annual ryegrass, and velvetleaf seedling length was caused by coumarin, juglone, nigericin, and plumbagin in the agar bioassay (Figure 2). Low and high doses of each compound caused little change in seedling length. The curvilinear regression equations were significant and described the dose response for each compound and plant species. Dose-response characteristics differed among phytotoxins. The ID_{50} values for nigericin, coumarin, juglone, and plumbagin were, respectively, 0.5, 2.0, 6.6, and 7.2×10^{-8} mole/seed for alfalfa; 1.2, 1.2, 7.4, and 4.0×10^{-8} mole/seed for annual ryegrass; and 1.7, 3.9, 3.6, and 5.6×10^{-8} mole/seed for velvetleaf. Nigericin exhibited the greatest phytotoxicity against seedling length, followed by coumarin, juglone, and plumbagin. Heisey and Putnam (1986) reported that

nigericin strongly inhibited radicle elongation of garden cress (*Lepidium sativum* L.). The agar bioassay is useful for determining the dose–response characteristics of a large variety of compounds.

Germination percentage was often positively correlated with reduced seedling length. Correlation coefficients between germination percentage and seedling length were 0.80 ($P = 0.01$), 0.91, and 0.61 for coumarin, juglone, and plumbagin with alfalfa; and 0.49, 0.94, and 0.89 with velvetleaf. In contrast, nigericin reduced alfalfa and velvetleaf seedling length but did not affect germination. Lower germination percentages were associated with reduced seedling length in the agar bioassay, even though only germinated seeds were considered in seedling length measurements. Phytotoxins that caused the largest reductions in germination percentage did not necessarily cause the largest reductions in seedling length.

Selectivity. The agar bioassay was capable of detecting differences in phytotoxicity among compounds and plant species. Alfalfa and velvetleaf germination were unaffected by cinmethylin. Cinmethylin (0.6×10^{-8} mole/seed) reduced the germination percentage of annual ryegrass by 70%. The germination of velvetleaf was reduced by 89% and 82% by 10×10^{-8} mole/seed of juglone and plumbagin, respectively (Table 1), whereas that of alfalfa was reduced by 26% and 39%, and annual ryegrass by 50% and 100% (Table 2). In contrast, 10×10^{-8} mole/seed coumarin reduced the germination of alfalfa and annual ryegrass by 84% and 100%, respectively, but did not reduce that of velvetleaf. Both naphthoquinones inhibited the germination of alfalfa and annual ryegrass more than that of velvetleaf, whereas coumarin did not reduce that of velvetleaf.

Cinmethylin reduced alfalfa and velvetleaf seedling length maximally by 30% and 18%, respectively, but that of annual ryegrass was reduced 97% by 1×10^{-9} mole/seed cinmethylin (Figure 3). Velvetleaf seedling length was inhibited more by plumbagin and juglone than was alfalfa (Figure 2). Juglone reduced alfalfa and velvetleaf seedling length by 66% and 95% at 1×10^{-7} mole/seed. Plumbagin reduced alfalfa and velvetleaf seedling length by 76% and 85% at 1×10^{-7} mole/seed. In contrast, the highest test doses of nigericin and coumarin reduced alfalfa seedling length by 86% and 94%, and velvetleaf by 78% and 73%.

Summary. The use of sensitive organisms such as garden cress (*Lepidium sativum* L.) in bioassaay is suggested as a way to identify toxic compounds. While useful in some applications, they may be too sensitive in others. The agar bioassay represents an alternative method able to test the effects of compounds on small-seeded agronomic, horticultural, or weed species, thereby emphasizing agricultural relevance.

An agar bioassay method is described that requires smaller quantities of plant growth inhibitors than the frequently used filter-paper bioassay to dem-

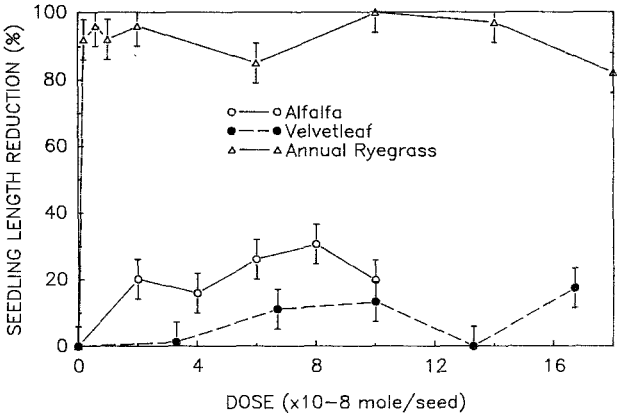

FIG. 3. The dose-response of cinmethylin on early seedling growth of alfalfa, annual ryegrass, and velvetleaf using the agar ± 1 SE.

onstrate comparable reductions in germination percentage. The use of agar as a growth medium in small tissue culture wells permits the accurate, reliable, and fast measurement of both the germination percentage and seedling length. The agar bioassay indicated that seedling length was more responsive than germination to compound phytotoxicity and was sensitive to selectivity among species. Dosage response dynamics of various compounds and plant species can be evaluated rapidly and analyzed statistically with the agar bioassay, permitting the rapid evaluation of poorly water-soluble organic compounds available in limited quantity.

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2,2′-OXO-1,1′-AZOBENZENE A Microbially Transformed Allelochemical from 2,3-Benzoxazolinone: I[1]

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Abstract—2,2′-Oxo-1,1′-azobenzene (AZOB), a compound with strong herbicidal activity, was isolated and characterized from a soil supplemented with 2,3-benzoxazolinone (BOA). A parallel experiment with 6-methoxy-2,3-benzoxazolinone (MBOA) yielded AZOB as well as its mono- (MAZOB) and dimethoxy- (DIMAZOB) derivatives. These compounds were produced only in the presence of soil microorganisms, via possible intermediates, I and II, which may dimerize or react with the parent molecule to form the final products. In the case of MBOA, it was shown that demethoxylation precedes the oxidation step. Although BOA and 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) were leached out of rye residues, there were no detectable amounts of the biotransformation products in the soil. When BOA was mixed with soil and rye residue, either under field conditions or in vitro, AZOB was detected. Levels of free BOA in the soil were greatly reduced by incubation with rye residue. AZOB was more toxic to curly cress (*Lepidium sativum* L.) and barnyardgrass (*Echinochloa crusgalli* L.) than either DIBOA or BOA.

Key Words—Allelopathy, benzoxazolinones, benzoxazinones, BOA, DIBOA, hydroxamic acids.

INTRODUCTION

Cubbon (1925) reported that a rye (*Secale cereale* L.) crop, grown simultaneously with a grape crop, reduced the grape's growth relative to a control. The high nutrient content of the soil and abundant water supply suggested that com-

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petition was not the cause of the reduced grape growth. Virtanen et al. (1957) isolated a cyclic hydroxamic acid, 2,3-benzoxazolinone, from rye seedlings. The same authors later isolated 6-methoxy-2,3-benzoxazolinone (MBOA) from wheat and corn (Virtanen et al., 1957) simultaneously with Loomis et al. (1957). These compounds were isolated and purified based on their biological activity against fungi (*Fusarium nivale*) and European corn borer (*Ostrinia nubilalis*). They also reported a glucoside precursor, 2-*O*-glucosyl-1,4(2H)-benzoxazin-3-one (GDIBOA) from rye, and a methoxylated analog, 2-*O*-glucosyl-7-methoxy-1,4-benzoxazin-3-one (GDIMBOA) from corn and wheat. Treatment of the glucoside with a crude enzyme preparation from rye caused hydrolysis to glucose and a second precursor, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) in rye and 7-methoxy-2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIMBOA) in corn and wheat (Virtanen and Hietala, 1960; Hietala and Virtanen, 1960). Hofman and Hofmanova (1969) showed that when intact tissue was carefully extracted, using liquid nitrogen as a fixative to prevent all enzyme activity, only the glucoside derivatives were extracted. This indicated that there was no free aglycone or benzoxazolinone in living intact tissue. Upon injury to the plant, β-glucosidases are released and rapidly hydrolyze the glucoside to the aglycone, which in turn decomposes in water to form the benzoxazolinone (Virtanen and Hietala, 1960). Naturally occurring benzoxazinones have also been reported from two other species, Job's tears (*Coix lachryma jobi*) (Koyama et al., 1955) and bears breech (*Acanthis mollis* L.) (Wolf et al., 1985). These are the only known instances of 1,4-benzoxazinone production in higher plants, although production by microorganisms is fairly common (Tipton et al., 1967).

Chou and Patrick (1976) studied the phytotoxic compounds released during the degradation of corn and rye residues. Plant material was combined with soil and allowed to decompose for up to 30 days. In the aqueous extracts of decomposing corn residue, they detected 18 phytotoxic compounds, mostly low-molecular-weight organic acids such as benzoic, phenylacetic, and 4-phenylbutyric acids, along with cinnamic acid derivatives such as ferulic, caffeic, *o*-coumaric, *p*-coumaric, *trans*-cinnamic, and vanillic acid. The cinnamic acid derivatives are frequently implicated in allelopathy by many species and are one of the fundamental groups of allelochemicals (Rice, 1984). Barnes and Putnam (1983, 1986, 1987) examined the allelopathic potential of residues and aqueous extracts of rye, and implicated the benzoxazinones, 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and 2,3-benzoxazolinone (BOA), cyclic hydroxamic acids, as the toxic agents produced by rye.

Patrick and Koch (1958) conducted a study of the toxic substances produced as a result of microbial decomposition of plant residues, including rye. They found that unless decomposition of the residues occurred, no toxic substances were present in the soil extract. If, however, decomposition had taken place, the extract of the soil–residue mixture was extremely toxic to the respi-

ration, oxygen uptake, and growth of tobacco (*Nicotiana tabacum* L.) seedlings (Patrick and Koch, 1958). They did not determine whether the toxins were of plant or microbial origin.

Once in the soil system, the benzoxazinones produced by rye would be susceptible to microbial transformation by various soil microbes. For the benzoxazinones to be involved in long-term allelopathic activity, they must be sufficiently resistant to such microbial transformations. Alternatively, if the parent compounds are metabolized, it is conceivable that biologically active metabolites may be involved in the overall process of allelopathy by rye residues. This paper describes the isolation and characterization of potent allelochemicals from soil previously supplemented with benzoxazinones and the detection of the levels of BOA and DIBOA in both residues and underlying soil over a period of time.

METHODS AND MATERIALS

Experimental. [^{1}H]- and [^{13}C]NMR analyses were performed on a Varian XL-300 spectrometer, 300 MHz for proton and 75 MHz for carbon. Electron impact mass spectral (EI-MS) analysis was done on a Hewlett Packard model 5895 quadruple mass spectrometer at 70 eV. Chemical ionization (CI) and high-resolution mass spectra (HRMS) were generated on a Jeol model HX-110 mass spectrometer. Ultraviolet (UV) absorption analyses were performed on a Gilford Response II ultraviolet spectrophotometer and infrared (IR) spectra, on a Perkin Elmer model 1170 FTIR spectrophotometer. Melting points were determined on a Thomas model 40 Micro Hot Stage apparatus and are uncorrected. HPLC analyses were performed on a Waters Radial Pak 8 mm ID $\times$ 10 cm, radially compressed C18 column (Waters Assoc., Division of Millipore Inc., Milford, Massachusetts), using KH_2PO_4 (0.01 M, pH 3.0)–acetonitrile (80:20 v/v). Unless specified, flash chromatography was performed on a silica gel column (Merck silica gel G, grade 60) and thin-layer chromatography, on silica gel plates (Merck silica gel G F-254, 0.250 mm layer). 2,3-benzoxazolinone (BOA) was obtained commercially from the Aldrich Chemical Co., Milwaukee, Wisconsin. Soil used was a Spinks loamy sand (Psammentric, hapludalf, sandy, mixed, mesic), collected from a field site at Michigan State University for use in the greenhouse, and stored in large bins under dry conditions for approximately one year. Soil was sterilized when necessary by autoclaving (1 hr on three successive days).

Spray Reagents for TLC Detection. DIBOA was detected on thin-layer plates with a spray reagent consisting of 5% $FeCl_3$ in 95% ethanol, acidified with conc. HCl. A spray reagent consisting of 1% ceric sulfate in conc. H_2SO_4 was used to detect BOA and MBOA.

Isolation of 6-Methoxy-2,3-benzoxazolinone (MBOA). Ten-day-old maize seedlings (Pioneer 3737) were harvested and frozen at −20°C overnight (592 g). The thawed plant material was homogenized in a Waring blender with distilled water (1.4 liters). This was kept at room temperature (1 hr) to ensure hydrolysis of the glucoside, 2-*O*-glucosyl-7-methoxy-1,4-benzoxazin-3-one (GDIMBOA) to 7-methoxy-2,4-dihydroxy-1,4-benzoxazin-3-one (DIMBOA) and refluxed (2 hr) to cause the conversion of DIMBOA to MBOA. After cooling, the extract was strained through cheesecloth and the filtrate was acidified with conc. HCl (pH 1.0). The acidified extract was filtered through Whatman No. 4 filter paper and extracted with diethyl ether (5 × 300 ml). The ether fraction was washed once with distilled water (300 ml), dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The crude extract thus obtained (720 mg) was initially purified by flash column chromatography ($CHCl_3$—MeOH 5 : 1) followed by TLC check with ceric sulfate detection. The fractions positive to ceric sulfate were pooled, and the solvent was removed *in vacuo*. This partially purified fraction (451.0 mg) was further chromatographed by TLC ($CHCl_3$—MeOH 6 : 1). The ceric sulfate-positive band was removed, eluted ($CHCl_3$—MeOH 2 : 1), and the solvent was removed by rotary evaporation. The colorless compound (129.8 mg) thus obtained was recrystallized from hexane–acetone, yielding needlelike crystals (70.8 mg) mp 144–148°C; UV (MeOH) 232.5 nm (9420), 290.0 nm (5050); IR (KBr) 3200, 1790, 1638, 1500, 1318, 1210, 1140, 1100, 1025, 970 cm^{-1}; [^{1}H]NMR (CD_3OD) δ 3.56 (3H, s, OCH_3), 7.80 (1H, d, J = 8.2 Hz), 7.65 (1H, d, J = 2 Hz), 7.58 (1H, d, J = 8.7 Hz); EI-MS, *m/z*: 165 (100, M^+), 150 (80).

Isolation of 2,4-Dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA). Seedlings of rye (Wheeler, 25 days old) were lyophilized at 15°C (101.5 g), homogenized in a Waring blender with distilled water (1 liter), and kept at room temperature (1 hr) to ensure enzymatic hydrolysis of the glucoside 2-*O*-glucosyl-1,4-benzoxazin-3-one (GDIBOA) to 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). After filtering through cheesecloth, the filtrate was heated in a water bath until the temperature of the extract reached 70°C. It was then cooled immediately in an ice bath. Coagulated components were removed by vacuum filtration using Whatman No. 1 filter paper. The filtrate was acidified with conc. HCl (pH 1.0) and extracted with diethyl ether (4 × 300 ml). The ether fraction was washed once with distilled water (300 ml), and the solvent was removed *in vacuo*. The crude extract thus obtained (355.8 mg) was initially purified by column chromatography ($CHCl_3$—MeOH 4 : 1), followed by TLC check with ferric chloride detection. The fractions positive to ferric chloride were pooled, and the solvent was removed at reduced pressure. This partially purified fraction (187.2 mg) was further chromatographed by TLC ($CHCl_3$—MeOH 6 : 1). The ferric chloride positive band was eluted ($CHCl_3$—MeOH 2 : 1), and removal of the solvent by rotary evaporation afforded a pale yellow residue (89.2 mg). This residue

was extracted with diethyl ether and filtered through a sintered glass filter (fine). Recrystallization from ether–cyclohexane afforded colorless needles (22.4 mg), mp 151–155°C; IR (KBr) 3400, 3200, 2380, 1670, 1590, 1280, 1220, 1040, and 750 cm^{-1}; [^{1}H]NMR (CD_3OD) δ 5.70 (1H, s), 7.35 (1H, dd, J = 1.0, 8.0 Hz), 7.10 (3H, m); HRMS, *m/z*: 181.0476 ($C_8H_7O_4N$, M^+).

Isolation of 2,2'-Oxo-1,1'-azobenzene (AZOB). 2,3-benzoxazolinone, (20 mg) was mixed thoroughly with soil (50 g), and the mixture was transferred to a 250 ml Erlenmeyer flask. Distilled water (5.0 ml) was added to the above and incubated at 26°C for 10 days. Soil for sterile controls was autoclaved. BOA and filter-sterilized (0.22-μm membrane) water (5.0 ml) were added aseptically to the soil, and the system was incubated at 26°C for 10 days. The control and experimental soil, as described above, were extracted with methanol (4 × 100 ml each). The methanol extract of the control soil was pale yellow, while the experimental extract was intensely orange. Both extracts were filtered, separately, through a sintered glass filter (fine), and the solvent was removed by rotary evaporation. A preliminary TLC analysis of the extracts indicated the presence of an orange band, only in the experimental soil. This orange compound (19.8 mg) was purified by TLC ($CHCl_3$—MeOH—HCOOH 20:1:1). Repeated purification was carried out by TLC (toluene—EtOAc—NH_4OH 5:4:1) until the resulting dark red compound (1.8 mg) gave only one spot by TLC. Recrystallization from hexane–acetone yielded AZOB as orange-red needles, mp 223–228°C (decomp.); UV (MeOH) 237 nm (ϵ = 28,268), 432 nm (ϵ = 23,711); IR (KBr pellet) 760, 1574, 1587 cm^{-1}; [^{1}H]NMR (DMSO) δ 7.75 (2H, d, J = 8.4 Hz, H-6, H-6'), 7.55 (6H, m, H-3, -4, -5 and H-3', -4', -5'); [^{13}C]NMR (DMSO) δ 99.87 (d, ×2), 104.86 (d, ×2), 117.25 (d, ×2), 126.60 (d, ×2), 129.19 (s, ×2), 130.34 (s, ×2); EI-MS, *m/z*: 212 (100, M^+), 185 (60), 184 (33); CI-MS, *m/z*: 212 (100, M^+), 185 (28) 184 (18); HRMS, *m/z*: 212.0588 ($C_{12}H_8N_2O_2$, M^+); FAB-MS (+) *m/z*: 213 (M^++H).

A similar experiment with DIBOA was carried out as in the case of BOA, and the work-up and purification yielded two compounds that proved to be BOA and AZOB, as well as unreacted DIBOA.

Isolation of AZOB from Field Soil to which BOA was Added. A commercial sample of BOA (1.0 g) was mixed with the top 13 mm of soil in a small plot (1.22 m) at the location from which soil used for in vitro conversion of BOA to AZOB was obtained. A soil sample (390 g) was taken from the center of the plot after seven days and extracted with methanol as above (105.5 mg). Removal of the solvent at reduced pressure gave an orange residue. Analysis of this extract by TLC confirmed the presence of AZOB. A control plot of the same size and location, but not supplemented with BOA, was also extracted for comparison and showed no benzoxazinones or diazoperoxides present.

Isolation of 4-Methoxy-2,2'-oxo-1,1'-azobenzene (MAZOB) and 4,4'-Dimethoxy-2,2'-oxo-1,1'-azobenzene, (DIMAZOB). The experiment with

MBOA was carried out as in the case of BOA and work-up and purification yielded a single component. Recrystallization from hexane–acetone afforded MAZOB as red-orange needles, mp 218–220°C (decomp.); UV (MeOH) 231 nm (ϵ = 23,591), 450 nm (ϵ = 21,122); IR: 2860, 2830, 1650; [^{1}H]NMR (DMSO) δ 3.70 (3H, s, OMe), 7.74 (1H, J = 8.4 Hz), 7.64 (1H, d, J = 8.8 Hz), 7.52 (3H, m), 7.11 (1H, J = 2 Hz), 7.01 (1H, J = 6 Hz); EI-MS, m/z: 242 (100, M^+); FAB-MS, m/z: 243 (M^+).

A small amount of 4,4′-dimethoxy-2,2′-oxo-1,1′-azobenzene, (DIMAZOB) was detected by both EI- and FAB-MS at 272 (M^+) and 273 (M^++H), respectively.

An additional experiment was carried out in which a 1 : 1 mixture of BOA and MBOA was added to soil and incubated as above. Work-up, purification, and analysis by EI-MS gave AZOB, MAZOB, and DIMAZOB. EI-MS, m/z: 212 (72%), 242 (26%), and 272 (1.5%) respectively.

Microbial Ecology of Transformation Reaction. Several attempts were made to isolate the organism(s) responsible for the biotransformation of BOA. Soil (1 g) was mixed with physiological saline (0.85% NaCl, 9 ml) and vortexed. Serial dilutions (1 : 10) were then plated on NZ-amine agar (NZ-amine A, 3 g; agar 18 g, distilled water 1 liter). The plates were sealed in a polyethylene bag and incubated at 25°C. After 72 hr, individual colonies were removed and inoculated into screw-capped test tubes containing sterile soil (5 g), and BOA (2 mg). These were wet with sterile distilled water (0.5 ml) and incubated as above.

Leaching Experiments with Rye Residue. Rye seedlings (Wheeler, 35 days old) were harvested at the soil level (900 g), placed on a flat (25 × 50 cm) of soil and watered over the top at 72-hr intervals. Residue and underlying soil samples were taken at 0, 1, 2, 3, 6, 10, 17, and 27 days, respectively. Methanol extracts (4 × 100 ml) of the soil samples were stored at −20°C. The lyophilized residue samples were homogenized in a Waring blender with distilled water (300 ml), kept at room temperature (1 hr), and strained through cheesecloth. The pH of the extract was adjusted to 1.0 with conc. HCl. The acidified extract was filtered through Whatman No. 1 filter paper under vacuum and extracted with diethyl ether (4 × 100 ml). The solvent was removed in vacuo and the dried samples were stored at −20°C.

Several experiments were also conducted in which lyophilized rye residue (10 g) was cut into 2 cm pieces and mixed into dry soil (1 kg) with distilled water (100 ml). Three such systems were set up in 2-liter beakers, each comprising a sample, that was extracted at 24, 72, and 240 hr respectively. Soil and residue were separated after drying at 45°C (1 hr). Methanol extraction of soil (3 × 500 ml) and removal of the solvent yielded dry samples that were stored at −20°C. The lyophilized residue was homogenized in a Waring blender with

distilled water (200 ml), kept at room temperature (1 hr), strained through cheesecloth, and acidified with conc. HCl (pH 1.0). The extracts were processed as above and stored at −20°C. All samples were analyzed simultaneously. Preliminary analysis of the samples was carried out by TLC ($CHCl_3$–MeOH 5 : 1). DIBOA and BOA were detected with the ferric chloride and ceric sulfate spray reagents, respectively. Samples that did not contain BOA or DIBOA by TLC were not analyzed further. Samples containing BOA or DIBOA were partially purified by TLC ($CHCl_3$—MeOH 5 : 1) and analyzed by HPLC. Both compounds were quantified respective to external standards by monitoring at 230 nm.

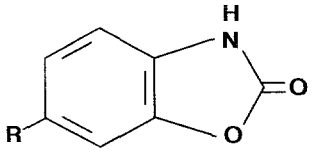
An additional experiment was conducted in which BOA (10 mg), rye tissue (5 g fresh), and distilled water (2.5 ml) were added to soil (25 g) and incubated at 26°C (10 days). An identical system, lacking residues, was used as a control.

Herbicide Bioassays. Phytotoxic activity was evaluated in terms of seedling growth inhibition. Stock solutions (1 mg/ml methanol) of purified compounds were applied to Whatman No. 1 filter paper in plastic Petri dishes (60 × 15 mm) to obtain doses ranging from 6.25 to 200 g/dish. Pure methanol was also added to a control plate. The plates were left open to allow the methanol to evaporate completely (20 min). Ten indicator seeds (curly cress and barnyardgrass) were then placed on the filter paper and distilled water (1.5 ml) was added. The plates were wrapped with parafilm to prevent drying and incubated in the dark (26°C for 72 hr). After incubation, seedling root length was measured and compared to the control. The experiments were designed as randomized complete blocks, with three replicates.

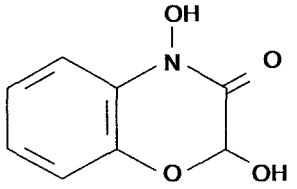
RESULTS AND DISCUSSION

AZOB (2,2′-oxo-1,1′-azobenzene), isolated as a biotransformation product of 2,3-benzoxazolinone (BOA) (Figure 1) from nonsterile soil, gave the molecular formula of $C_{12}H_8N_2O_2$ by HRMS. The doublet at 7.75 ppm, integrated for two protons was assigned to the 6 and 6′ hydrogens ortho to the nitrogen in the aromatic ring. The only other signal observed in the [^{1}H]NMR spectrum was a multiplet at 7.55 ppm, integrated for six protons. The [^{13}C]NMR spectrum of AZOB gave only six signals for 12 carbon atoms, indicating a symmetrical structure for this molecule. The strong IR absorption at 760 cm^{-1}, indicative of an aromatic system with four adjacent hydrogens, was in full agreement with the NMR data and confirmed the proposed structure (Figure 2).

Incorporation of DIBOA into soil also resulted in the formation of AZOB. Addition of methoxylated BOA (MBOA) gave MAZOB and DIMAZOB, characterized by spectral methods (Figure 2). It is interesting to note that the most

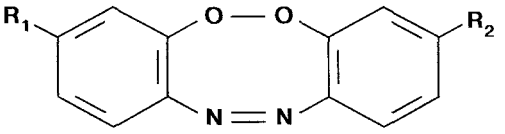


BOA: R = H
MBOA: = OMe



DIBOA

FIG. 1. Benzoxazolinones and benzoxazinones isolated from rye or corn plants.

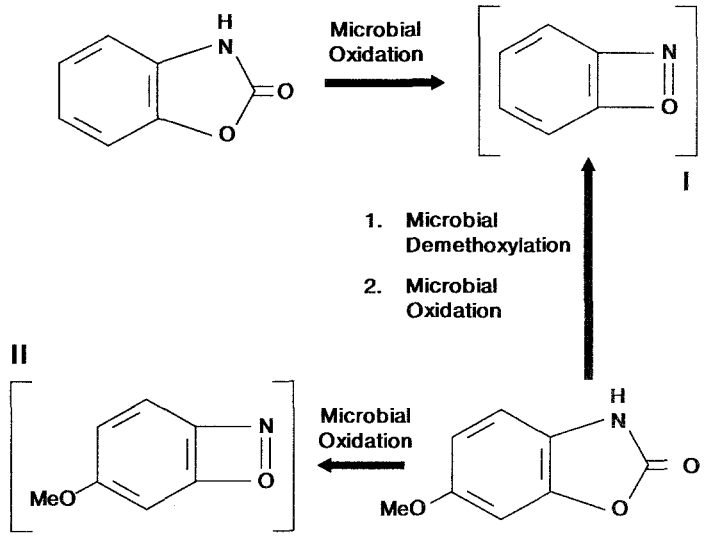


AZOB: $R_1 = R_2 = H$
MAZOB: $R_1 = H, R_2 = OMe$
DIMAZOB: $R_1 = R_2 = OMe$

FIG. 2. Azobenzene transformation products produced from benzoxazolinone by soil microbes.

abundant product of MBOA transformation is MAZOB (95%), with DIMAZOB present as a minor species, as evident by mass spectral data. Trace amounts of AZOB were also present in the above mixture.

Formation of AZOB in the soil could be the result of an intermediate **I** (Scheme 1), produced enzymatically from BOA, which in turn reacts with another molecule of BOA. Alternatively, this intermediate could undergo dimerization to afford AZOB. In the case of MBOA, the major product was

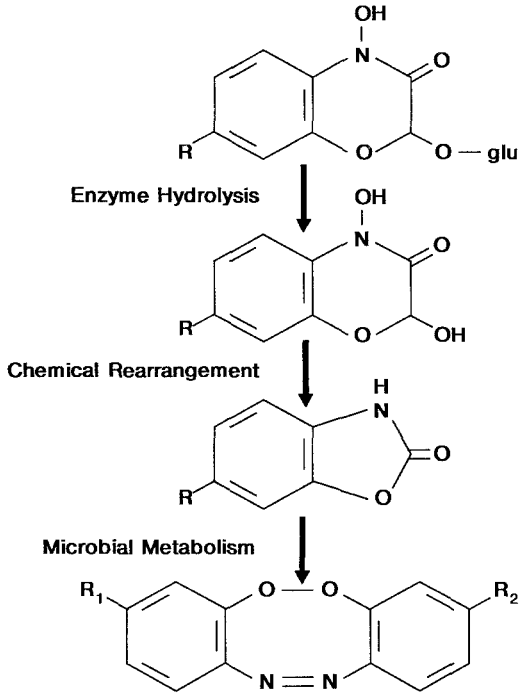


SCHEME 1. Probable transformation scheme for conversion of BOA to AZOB.

MAZOB, a monomethoxy analog of AZOB. This indicated that prior to the enzymatic oxidation leading to intermediate **II** (Scheme 1), there is a demethoxylation step involved. That is, an enzymatic demethoxylation, followed by enzymatic oxidation, would also yield intermediate **I**, which in turn would react with MBOA, to give MAZOB. The dimethoxy derivative (DIMAZOB) should result from the reaction of intermediate **II** with MBOA or from dimerization of intermediate **II**. The experiment with equal amounts of BOA and MBOA gave AZOB as the major product (72%), MAZOB (26%), and DIMAZOB in very small quantity (1.5%). Therefore, it is evident that intermediate **II** is less abundant than intermediate **I** and that the demethoxylation occurs prior to the enzymatic oxidation. Based on these results, it is possible to extend the breakdown scheme for benzoxazinones proposed by Virtanen et al. (1960) (Scheme 2).

Addition of BOA or MBOA into sterile soil did not produce any of the azobenzenes, suggesting that these compounds are produced by soil microbes. To isolate the total microbial population, a saline extract of the soil was added to soil containing BOA, and resulted in production of AZOB after incubation for seven days.

Experiments to isolate the organism(s) responsible for the transformation were inconclusive. None of the colonies isolated to date was capable of transforming BOA. It appears as if the transformation reaction does not provide a



SCHEME 2. Extension of the degradative scheme for BOA proposed by Hietala and Virtanen (1960).

selective advantage to those microbes capable of carrying it out. That is, the reaction is probably not an energy-yielding reaction, but perhaps, instead, is catalyzed by an extracellular oxidase enzyme. Production of AZOB was also demonstrated in the field, using commercial BOA, indicating that these organisms are present in the environment. Although trace amounts of BOA and DIBOA were isolated from the soil in laboratory leaching experiments using rye residue, AZOB was apparently not present in this soil. In order to verify this result, an in vitro experiment with rye residue and BOA was conducted and the products were quantified. Only trace amounts of AZOB were observed by TLC from the soil containing BOA and fresh rye residue. It was also interesting to note that most of the added BOA in the residue sample (92%) had disappeared. The control sample, containing no residue, produced the normal amount of AZOB as mentioned earlier, yet contained approximately 9.7 times as much of the added BOA as did the residue treatment. These results indicated that either some enzymatic inhibitors had leached from the rye residue into the soil, or that the leachate had killed the microbe(s) responsible for the conversion.

Since the microbes responsible for the production of azobenzenes have not yet been identified, it is difficult to argue that the leachate has killed these organisms. Chemical reaction between BOA and the leachate is a possible explanation for the diminished production of AZOB, as well as the disappearance of most of the added BOA. The above results were repeated in large-scale experiments as well.

Barnes and Putnam (1987) found dicotyledonous species to be approximately 30% more sensitive to BOA and DIBOA than were monocotyledonous species. Initial assays, conducted with garden cress and barnyardgrass as indicators, indicated a high degree of toxicity of AZOB to radicle elongation of both species (Figure 3). AZOB was much more toxic than either BOA or DIBOA. This compound could contribute to the overall toxicity of rye residues, for which Barnes could only attribute a small portion (12%) to the hydroxamic acids.

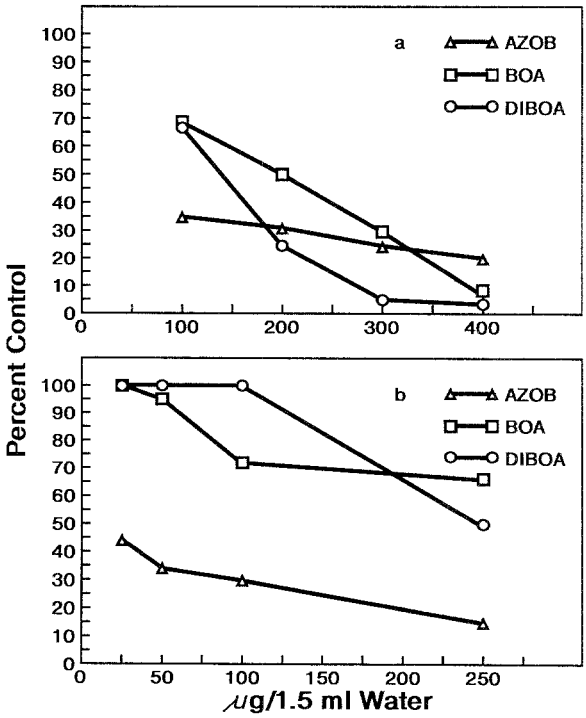


FIG. 3. Relative toxicities of 2,2′-oxo-1,1′-azobenzene and the parent benzoxazinones, BOA and DIBOA, as measured by inhibition of radicle elongation of (a) garden cress and (b) barnyardgrass. All points are significantly different from control at the 0.05 level by LSD.

Our results suggest a role for soil microorganisms in the overall process of allelopathy by rye. The primary allelochemicals produced by rye, BOA, and DIBOA have been shown to be present in the soil by in vitro leaching experiments. Once in the soil, these compounds undergo microbial transformation, resulting in the diazoperoxide, AZOB. Although these compounds were not detected in either *in vivo* or *in vitro* experiments using rye residues, the biological activity of AZOB clearly demonstrates the potential role of these compounds in allelopathy if a suitable microbial population is present in soil to perform their synthesis.

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INSECT ANTIFEEDANT ACTIVITY ASSOCIATED WITH COMPOUNDS ISOLATED FROM SPECIES OF *Lonchocarpus* AND *Tephrosia*

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Abstract—The antifeedant activity of a series of 21 chalcones, flavanes, and flavanones isolated from the genera *Lonchocarpus* and *Tephrosia* (Leguminosae) was assessed by behavioral and electrophysiological bioassays against larvae of *Spodoptera littoralis* and *S. exempta*. The antifeedant activity is related to the molecular structure of the compounds, and possible modes of interaction with the insect taste receptors are discussed.

Key Words—*Spodoptera littoralis*, *Spodoptera exempta*, Lepidoptera, Noctuidae, *Lonchocarpus*, *Tephrosia*, antifeedant, feeding behavior, gustatory codes.

INTRODUCTION

Many species of the plant family Leguminosae are protected from attack by insects by the presence of allelochemicals that have toxic or antifeedant properties, or both, against a range of insects that are major pests of crop plants (Birch et al., 1989). This article is one of a series in which we are assessing the antifeedant activity of naturally occurring compounds and synthetic com-

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pounds derived from them (Simmonds and Blaney, 1984; Simmonds et al., 1985; Blaney et al., 1987, 1988). In doing so, we are also exploring facets of insect feeding behavior and, in particular, the sensory physiology of the receptor systems principally deployed by insects in host plant selection.

The compounds tested in this paper are flavanones, flavanes, and chalcones, belonging to a homogeneous group of C-15 derivatives characterized by the presence of one isoprenyl group. These compounds may represent intermediate stages in the biosynthesis of the insect-toxic rotenoids, which occur in the genera *Lonchocarpus* and *Tephrosia*, belonging to the family Leguminosae, from which the present compounds are isolated or derived (Delle Monache et al., 1973, 1978, 1986; Goncalves de Lima et al., 1975; Lupi et al., 1975; Cuca Suarez et al., 1980).

In studying the structure–function relationships of plant compounds with antifeedant activity, we have found it constructive to compare the behavioral and electrophysiological responses of lepidopterous larvae that differ in their feeding specificity (Simmonds and Blaney, 1984; Blaney et al., 1987, 1988). In this article we use the oligophagous larvae of *Spodoptera exempta* (Walker), which feed almost exclusively on Gramineae, and the polyphagous larvae of *Spodoptera littoralis* (Boisduval).

METHODS AND MATERIALS

Insects

Larvae of *S. littoralis* were reared on a bean-based diet (Dimetry, 1976) and those of *S. exempta* on wheat seedlings. All the insects were reared at Birkbeck College in a 16 : 8 hr light–dark photoperiod at 26° ± 1°C. Larvae, 24–36 hr into the final stadium, were deprived of food for 4 hr before being used in a bioassay.

Chemicals

The compounds used and their origins are listed in Table 1, and their molecular structures are shown in Figure 1. In order to facilitate comparison between the assays, the same test solutions were used for both behavioral and electrophysiological assays. Thus, since the electrophysiological assay requires the presence of an electrolyte, all solutions contained 0.05 M sodium chloride. The compounds were first dissolved in 95% ethanol, then made up with distilled water so that the final concentration of ethanol was never more than 0.5%. At that concentration, these solvents do not affect the sensory responses (Simmonds and Blaney, 1984, and unpublished results). The test compounds were presented at the concentrations shown in Tables 2 and 3 below.

TABLE 1. NAME AND ORIGIN OF PLANT FLAVONOIDS

Compound	Name	Origin	Reference[a]
1	2',4'-Dihydroxychalcone	[b]	1
2	4'-O-Prenyl-chalcone	*Lonchocarpus neuroscapha*	1
3	3'-Prenyl-chalcone	*Lonchocarpus neuroscapha*	1
4	Derricin	*Lonchocarpus neuroscapha*	1
5	Lonchocarpin	*Lonchocarpus neuroscapha*	1
6	6-Cinnamoyl-5-hydroxy-2, 2-dimethylchroman	[b]	2
7	6-Cinnamoyl-7-hydroxy-2, 2-dimethylchroman	[b]	2
8	4'-Hydroxyisoderricin	synthetic	3
9	Methylhildgardtol A	*Tephrosia hilbrandtii*	4
10	Methylhildgardtol B	*Tephrosia hilbrandtii*	4
11	7-Hydroxyflavonone	[b]	1
12	7-O-Prenyl-flavonone	*Lonchocarpus neuroscapha*	1
13	8-Prenyl-flavanone	[b]	1
14	7-O-Methyl-8-prenylflavanone	[b]	1
15	Isolonchocarpin	*Lonchocarpus eriocaulinalis*	5
16	Dihydroisolonchocarpin	[b]	2
17	6″,6″-Dimethyl-2H-pyrano (2″,3″,7,6)-flavanone	[b]	2
18	5-Hydroxyisoderricin (7-methyl-grabanin)	*Tephrosia villosa*	6
19	7-O-Methyl-8-(3-methyl butadienyl)-flavanone	*Tephrosia purpurea*	6
20	5-Methoxyisolonchocarpin	*Tephrosia vogelii*	6
21	3-Hydroxyisolonchocarpin	*L. eriocaulinalis*	5

[a] References: 1 = Goncalves de Lima et al., 1975; 2 = Delle Monache et al., 1973; 3 = Lupi et al., 1975; 4 = Delle Monache et al., 1986; 5 = Delle Monache et al., 1978; 6 = Cuca Suarez et al., 1980.
[b] Derived from plant compound.

Bioassay

Four different bioassays were used to test the compounds.

Dual-Choice Test (A). Individual larvae were confined for up to 5 hr in a Petri dish (9 cm diameter) containing two glass-fiber disks (GFD). Both disks (Whatmans GB/A, 1 cm) were treated with 50 μl of a solution containing 0.05 M sucrose (to make the disk palatable) and 0.05 M sodium chloride. The application of this amount of solution to each disk ensures that the compound is evenly distributed over the disk. One disk served as the control (C); the other, the treatment disk (T), received additionally, when dry, a 50-μl aliquot of the

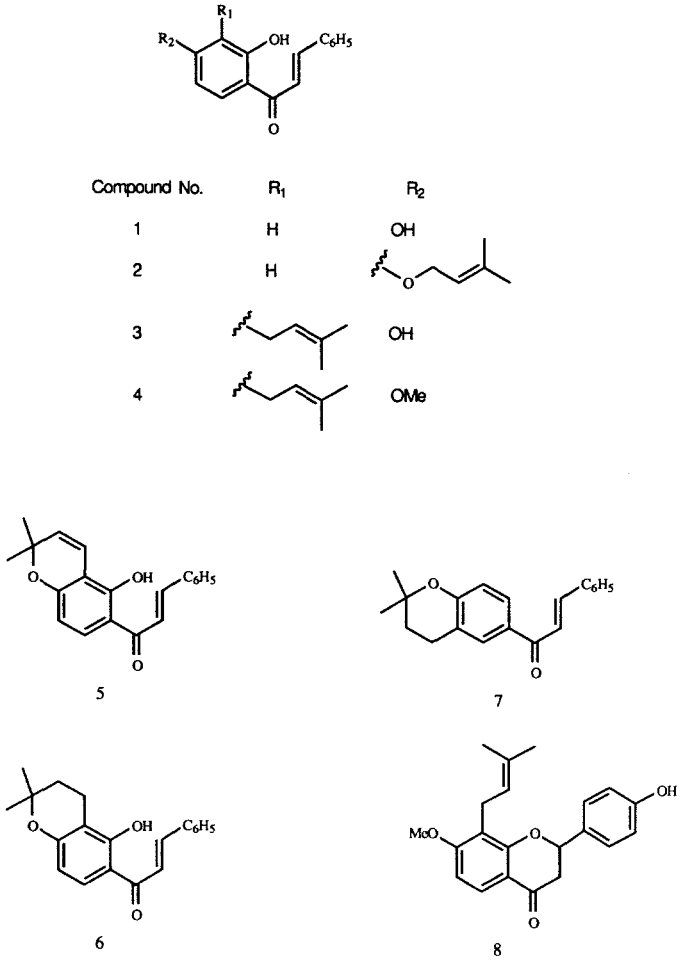
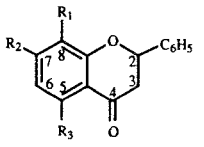
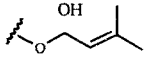
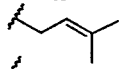
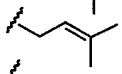
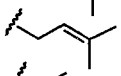
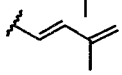


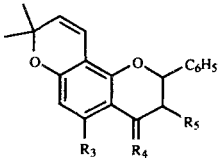
FIG. 1. Structures of the 21 compounds tested.

test solution. The disks were dried and weighed before being presented to the larvae. The experiments were terminated after 5 hr, or when 50% of any disk had been eaten. The disks were then weighed, and the amount of each disk eaten was used to calculate the antifeedant index $[(C - T)/(C + T)]\%$. This index distinguishes phagostimulants (negative values) and deterrents (positive values).

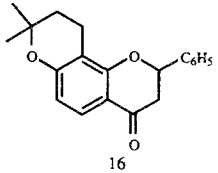
Dual-Choice Leaf Disk Test (B). This test was similar to test A, but leaf disks were used instead of GFD. For *S. littoralis* disks (1 cm diameter) of cotton leaves were used, and for *S. exempta* an equivalent area of wheat leaf blade was used. The treatment leaf disks (T) were painted with a 30-μl aliquot of test



Compound No.	R_1	R_2	R_3
11	H	OH	H
12	H		H
13		OH	H
14		OMe	H
18		OMe	OH
19		OMe	H



Compound No.	R_3	R_4	R_5
15	H	O	H
10	OMe	H,OMe	H
20	OMe	O	H
21	H	O	OH



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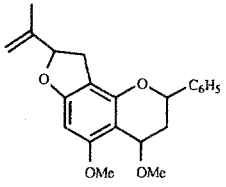

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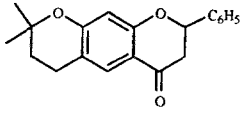

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FIG. 1. Continued.

solution, and the control disks were treated with solvent solution only. Both solutions contained 0.1% Tween 80. The bioassay was for 1 hr or until 50% of either disk was eaten. The amount eaten was assessed visually to the nearest 5% by comparing the remaining leaf material with a template of the original plant material. The antifeedant index was calculated using the percentage eaten.

No-Choice Leaf Disk Test (C). Disks were painted with the test solution,

as in test B, and each larva was left with a disk for 1 hr (no larva consumed more than 70% of a disk).

Electrophysiological Bioassay (D). A description of this bioassay can be found in Blaney et al. (1987). Briefly, the paired maxillary sensilla styloconica are stimulated with a range of solutions using a standard tip-recording technique (Hodgson et al., 1955). Each larva was stimulated with the control solution, 0.05 M sodium chloride, and up to five of the test solutions. All compounds were tested on three to five larvae, with one to five replications for each lateral and medial styloconic sensillum. All compounds, except 8 and 21 (Table 1), were tested against both species in the dual-choice GFD bioassay at 100 ppm. However, in the other bioassays, because of the limited amounts of compounds available, the concentration used and the number of compounds tested varied.

RESULTS

The results of the dual-choice bioassay with GFD are given in Table 2. The antifeedant effect was generally much greater against *S. exempta* than against *S. littoralis*. The 4-methoxyflavanes (9 and 10) and the flavanones (11, 12, 13, and 16) were significantly more active against *S. exempta* than *S. littoralis*, at 100 ppm. Fourteen of the 20 compounds tested at 100 ppm against *S. exempta* had significant antifeedant activity, and 13 of them were still active at 10 ppm. The flavanones were more active than the chalcones, indeed, only flavanone (17) did not have a significant level of activity against *S. exempta.*

Of the 11 compounds available in sufficient quantity to be tested at 500 ppm against *S. littoralis*, six (1, 5, 6, 12, 13, and 14) had significant antifeedant activity, but only two of them (5 and 14) were still active at 100 ppm. Of the other compounds tested at 100 ppm, five (4, 15, 18, 19, and 20) had significant antifeedant activity, and four of them (4, 15, 19, and 20) were still active at 10 ppm. Of these four, the three flavanones (15, 19, and 20) were more active than the chalcone (4). Flavanone (21) was the only compound to significantly increase feeding.

The results of the dual-choice test with leaf material are given in Table 3. The two chalcones (5 and 6) and all the flavanones (11, 12, 13, 14, and 17) tested against *S. exempta* were significant antifeedants at 100 ppm. Of the six chalcones tested against *S. littoralis*, three (1, 5, and 6) were significantly active at 500 ppm, and two of them (5 and 6) retained activity at 100 ppm. Chalcone (7) was active at 100 ppm but not at 500 ppm. Of the five flavanones tested, only one (14) was active. Generally, the antifeedant activity on the leaf disks was lower than on the GFD (see statistics in Table 3). There was one exception, however: with *S. littoralis*, chalcone (6) was more active on leaf disks than on GFD.

TABLE 2. ANTIFEEDANT INDEX $[(C - T)/(C + T)]\%$ OF TEST COMPOUNDS (MEAN ± SEM) IN DUAL-CHOICE TEST WITH GLASS FIBER DISKS: CONTROL (C) VERSUS TREATMENT (T)[a]

Compounds	Spodoptera exempta[b]		*Spodoptera littoralis*[b]			Betw. sp.[c]
	100 ppm	10 ppm	500 ppm	100 ppm	10 ppm	
Chalcones						
1	11 (13.4)		59 (12.9)**	22 (8.6)		
2	10 (8.4)		−3 (22.7)	−15 (21.6)		
3	11 (16.5)		−40 (17.9)	11 (8.1)		
4	72 (6.4)**[d]	54 (8.4)**		75 (19.7)***	56 (21.4)*	
5	55 (11.4)**	39 (6.3)*	33 (24.4)*	43 (16.2)*		
6	52 (8.4)**	41 (8.3)*	68 (11.5)**	32 (31.3)		
7	13 (8.6)		24 (24.3)	48 (25.5)		
Flavanes						
9	36 (9.3)*	13 (3.8)		19 (16.1)	38 (17.7)	+
10	72 (6.4)**	65 (8.4)**		39 (11.6)	27 (16.4)	+
Flavanones						
8				−14 (18.5)		
11	73 (6.5)**	59 (2.5)**	10 (19.6)	17 (14.3)		+
12	74 (11.9)**	36 (8.4)*	29 (15.6)*	25 (12.8)		+
13	79 (6.4)***	34 (8.6)*	41 (8.8)**	11 (22.3)		+
14	65 (11.3)**	29 (8.7)*	42 (13.1)*	73 (17.0)**		
15	74 (8.4)**	59 (4.6)**		88 (5.6)***	87 (10.4)**	
16	75 (11.3)**	42 (8.4)**		23 (16.8)	33 (13.6)	+
17	13 (8.6)	3 (8.4)	6 (2.3)	13 (18.3)		
18	71 (8.2)**	42 (11.3)*		53 (12.4)*	2 (18.4)	
19	69 (8.5)**	42 (11.4)*		43 (19.2)*	85 (8.9)***	
20	80 (11.3)***	35 (14.9)*		64 (7.4)**	59 (17.6)**	
21				−83 (16.9)***		

[a] See text for details.
[b] No. of replications = 20–40.
[c] Betw. sp. + = significant difference between the species of *Spodoptera* at 100 ppm (Mann-Whitney test; $P < 0.05$).
[d] *, **, *** indicate significant difference from control at 0.05, 0.01 and 0.001 levels, respectively; t test on transformed data ($x + 0.05$).

The results of the no-choice leaf disk bioassay have been correlated with those of the electrophysiological bioassay and are presented in Figures 2 and 3. Of the 11 compounds tested, those most active behaviorally against *S. exempta* were the flavanones 11, 12, 13, and 14, and against *S. littoralis* the most active were the chalcones 2, 6, and 7.

In the electrophysiological bioassay, the responses of the two species differ

TABLE 3. ANTIFEEDANT INDEX $[(C - T)/(C + T)]\%$ OF TEST COMPOUNDS (MEAN ± SEM) IN DUAL-CHOICE TEST WITH LEAF DISKS: CONTROL (C) VERSUS TREATMENT (T)[a]

Compound	*Spodoptera exempta*[b]	*Spodoptera littoralis*[b]		Betw. sp.[c]
	100 ppm	500 ppm	100 ppm	
Chalcones				
1		53 (11.7)**	22 (16.8)	
2		0 (5.5)	−23 (2.5)	
3		11 (11.2)[e]	7 (8.9)	
5	41 (16.5)*[d]	32 (5.8)*	41 (10.7)*	
6	42 (8.1)*	50 (4.4)**	73 (3.8)**[e]	+
7		8 (8.8)	50 (4.8)**	
Flavanones				
11	31 (8.6)*[e]	−2 (10.4)	24 (11.5)[e]	
12	34 (6.4)*[e]	12 (24.2)	19 (17.7)	+
13	34 (4.7)*[e]	25 (15.1)[e]	2 (18.7)	+
14	24 (3.9)*[e]	43 (12.7)*	57 (15.7)*[e]	
17	34 (6.5)[e]	−2 (13.4)	24 (18.3)	

[a] See text for details.
[b] No. of replications: 10–20.
[c] Betw. sp. = significant difference between the species of *Spodoptera* at 100 ppm (Mann Whitney test; $P < 0.05$).
[d] *, ** indicate significant difference from control at 0.05 and 0.01 levels, respectively; t test on transformed data ($x + 0.05$).
[e] Significant difference from results with GFD (Table 2) (Mann Whitney test; $P < 0.05$).

in important respects. In *S. exempta*, a single neuron in the medial sensillum responded to the test compounds, and its firing rate was significantly negatively correlated with the amount of plant disk eaten (Figure 2; $r = -0.9784$; $P < 0.001$); the firing rate increased as the amount eaten decreased. In *S. littoralis* the compounds stimulated one, two, or three neurons in the medial sensilla (identified as neurons A, C, and D, see discussion and Blaney et al., 1987, 1988). The output of each neuron was identified by the shape and height of its action potentials. There was found to be a significant correlation between the total firing rate of the medial sensilla during the first second of stimulation with the compounds and the amount of plant disks eaten (Figure 3; $r = 0.8775$; $P < 0.001$). An interesting result from this experiment is that the more unpalatable the test solution, the lower the firing rate elicited.

The compounds did stimulate one to two neurons in the lateral sensillum of both species, but their total response was low (5–15 impulses/sec) and sporadic. As this response did not correlate with the feeding behavior, it has been excluded.

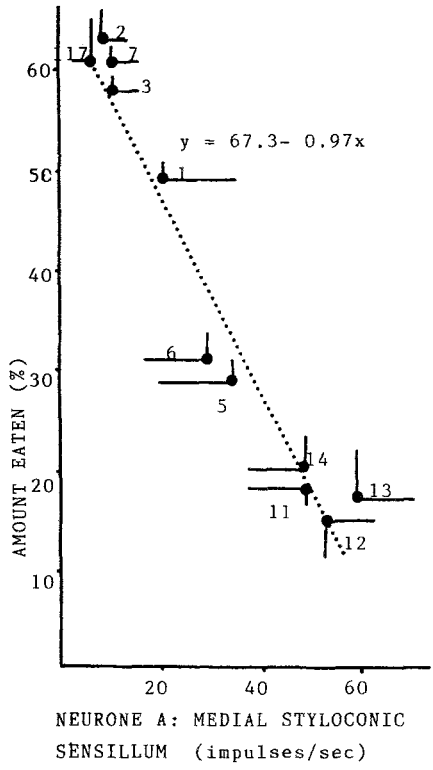

FIG. 2. The relationship between the amount eaten by larvae of *S. exempta* in the no-choice bioassay, using leaf disks coated with 30 μl of test compound at 100 ppm, and the firing rate of neuron A in the medial styloconic sensillum when stimulated with the same test compounds. Numbers = compounds in Figure 1: range bars = SEM; replications: behavior = 10–15; electrophysiology = 3–5 larvae.

Clearly, the sensory code for taste quality mediated by the styloconic sensilla is either more complex or more obscure in *S. littoralis* than in *S. exempta*. A further analysis of the performance of individual neurons in *S. littoralis* has been undertaken (Table 4 and Figure 4). Compounds 2, 3, 11, 12, 13, and 17 stimulated neuron C more than neuron A, and neuron A was stimulated more than neuron C by compunds 1, 5, 6, 7, and 14 (Table 4). Another neuron, D, which was stimulated strongly by the control solution, sodium chloride, was active in all the recordings, although it represented a low percentage of the total response. The response of neuron A, when taken as a percentage of the total firing of the medial sensillum during the first second of stimulation, correlates significantly with the antifeedant index from the GFD bioassay (Figure 4; r =

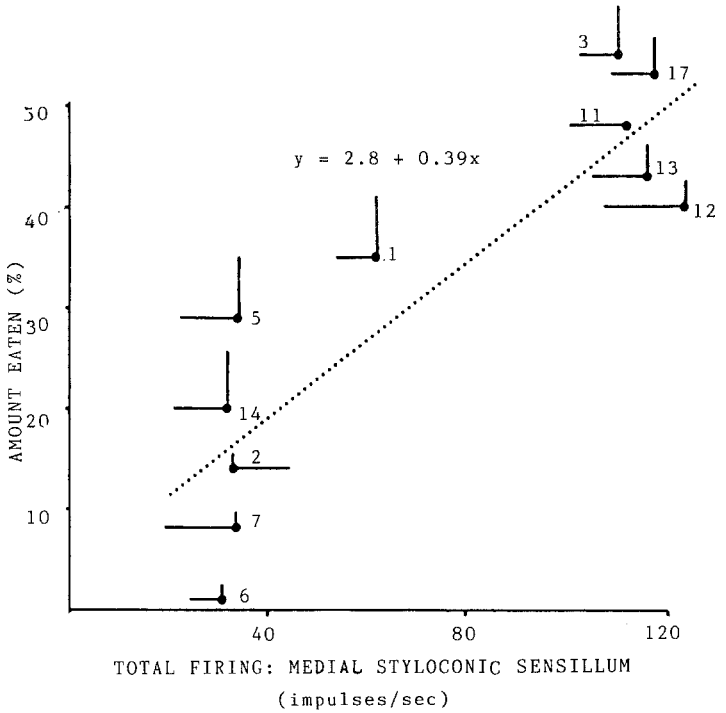

FIG. 3. The relationship between the amount eaten by larvae of *S. littoralis* in the no-choice bioassay, using leaf disks coated with 30 μl of test compound at 100 ppm, and the total firing rate of the medial styloconic sensillum when stimulated with the same compound. Numbers = compounds in Figure 1: range bars = SEM; replications: behavior = 10–15; electrophysiology, see Table 4.

0.8243; $P < 0.001$), the antifeedant index from the leaf disk bioassay ($r = 0.7610$; $P < 0.001$), and the amount eaten in the no-choice ($r = 0.5576$; $P < 0.01$).

DISCUSSION

In general, the antifeedant effect of the compounds was greater with the oligophagous *S. exempta* than with the polyphagous *S. littoralis*. A similar difference in susceptibility has been found previously with these species, both with natural plant compounds and with synthetic derivatives of them (Blaney and Simmonds, 1983; Simmonds and Blaney, 1984; Simmonds et al., 1985; Blaney et al., 1987).

TABLE 4. RESPONSES OF NEURONS IN MEDIAL STYLOCONICA SENSILLA OF *Spodoptera littoralis* STIMULATED WITH A RANGE OF CHALCONES AND FLAVANONES[a]

Compound	No. of insects	No. of stimulations	Neuron response impulses per sec [mean (SEM)] A	C	D
NaCl	45	259	14.1 (8.21)	2.1 (1.41)	31.4 (6.41)
Chalcones					
1	5	14	35.2 (2.72)	25.8 (2.52)	3.2 (0.80)
2	5	13	7.2 (1.16)	23.2 (2.06)	3.4 (0.40)
3	7	20	33.8 (8.22)	71.5 (9.75)	5.0 (2.17)
5	6	11	23.8 (1.68)	7.5 (0.68)	2.8 (1.34)
6	5	10	19.4 (1.40)	7.6 (0.93)	4.2 (0.49)
7	5	12	17.6 (1.68)	15.2 (2.22)	2.0 (0.78)
Flavanones					
11	6	18	29.7 (3.82)	71.3 (8.69)	11.3 (2.89)
12	6	14	34.1 (5.77)	83.7 (5.34)	6.8 (2.08)
13	6	14	38.1 (2.81)	69.2 (10.75)	4.3 (1.33)
14	5	11	24.8 (3.62)	5.6 (1.29)	2.0 (0.93)
17	6	14	20.8 (3.16)	86.7 (4.56)	8.8 (3.83)

[a]Conc. 100 ppm in NaCl 0.05 M.

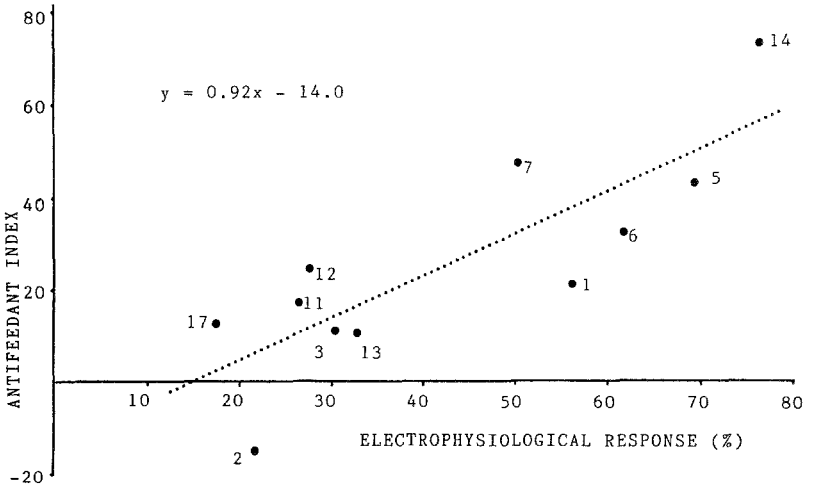

FIG. 4. The relationship between the antifeedant index $[(C - T)/(C + T)]\%$ with larvae of *S. littoralis* in the glass fiber disk bioassay and the electrophysiological response, represented by the firing rate of neuron A given as a percentage of the total firing in the medial styloconic sensillum. Numbers = compounds in Figure 1.

The type of feeding bioassay used affects the results obtained and influences particularly the assessment of a compound as an antifeedant with potential for practical application. Leaves of host plants are generally more palatable to phytophagous insects than glass-fiber disks, even with sucrose added. It is therefore not surprising to find that, overall, the antifeedant activity was lower on leaf disks than on glass-fiber disks. This effect is important, and care should be taken when comparing activity obtained with different bioassays (Simmonds et al., 1985; Lewis and Van Emden, 1986; Blaney et al., 1987).

The fundamental moiety of all these compounds is the C6—C—C—C—C6 system found in chalcones, which may cyclize to give flavanones and other flavonoids including flavanes (Goodwin and Mercer, 1983). The 4-methoxy-flavanes (9 and 10) differ fundamentally from the flavanones and should be considered separately.

Consideration of antifeedant activity in relation to molecular structure indicates that the flavanones were generally more active than the chalcones. There are some interesting structure–activity relationships in which the same substitutions, in both the chalcones and flavanones, are associated with antifeedant activity. Thus the chalcones and their corresponding flavanones most active against both species are 4 and 14 and 5 and 15, respectively.

For *S. littoralis*, a comparison of the antifeedant activity associated with the flavanones 14 and 13 and chalcones 4 and 3 shows that the methoxy substitution at R2 (14 and 4) is more active than the hydroxy substitution at R2 (13 and 3). However, the picture is not so clear for *S. exempta*: in the chalcones, the methoxy substitution (4) is more active than the hydroxy substitution (3), but in the flavanones 13 and 14 both are equally active substitutions. Differences such as this suggest that, while both species have neurons that respond to these compounds, the receptor sites on the relevant neurons of *S. exempta* differ in structure and/or function from those of *S. littoralis*.

In *S. littoralis* the presence of a double bond in the dimethyl chromene ring of flavanone (15) and chalcone (5) appears to be important in determining antifeedant activity because the reduced compounds 16 and 6 are less active. Within the angular 4-methoxy-flavanes (9 and 10), the isopropenylfuran ring in compound 9 is associated with poor antifeedant activity, whereas in compound 10 the dimethyl chromene ring with a double bond conjugated with the aromatic ring is associated with low but increased antifeedant activity. However, the chromene ring is also present in the active phagostimulant (21), although in the presence of an hydroxy substitution at R5.

The introduction in the molecule of a methoxy group at C-5 (compound 15 versus 20) does decrease activity in *S. littoralis* but not in *S. exempta*, whereas the introduction of a hydroxy group at C-3 makes the compound a phagostimulant for *S. littoralis* (21 versus 15).

An important and intriguing consideration in studies such as this is the way

in which information on the palatability status of compounds and mixtures is conveyed to the insect by means of the sensory code for taste quality. In this respect there are fundamental differences between the oligophagous *S. exempta* and the polyphagous *S. littoralis*.

When the maxillary styloconic sensilla of *S. exempta* and *S. littoralis* are stimulated with antifeedant compounds, responses may be evoked from both medial and lateral sensilla (Simmonds and Blaney, 1984; Blaney et al., 1985, 1987, 1988), but, most commonly, it is the response from the medial sensilla that is dose-dependent and correlates best with antifeedant activity. Each sensillum contains four neurons, and all four may be active during a given stimulation, but often only one neuron is entirely or predominantly responsible for the dose-dependent response. In order to discourage prejudgement of the roles and activities of these neurons, we have refrained from identifying them exclusively in terms of the stimulants to which they give the greatest magnitude of response (i.e., "sugar best" or "salt best" neuron) and have called them simply neurons A, B, C, and D. However, in *S. exempta* there is one neuron, neuron A, in the medial sensillum that responds to the majority of compounds that deter feeding, and we, like Ma (1976), refer to this neuron as the "deterrent" neuron.

In the present study with *S. exempta*, the compounds all stimulate predominantly the deterrent neuron in the medial sensillum. However, it is possible that more than one receptor site is involved. Examination of Figure 2 suggests that the compounds fall into two categories. Flavones 11, 12, 13, and 14 elicit similar firing rates, and the resulting behavior is similar, as seen by the amount eaten in the no-choice bioassay or by the antifeedant index in the GFD bioassay. Flavanone 17 and chalcones 1, 2, 3, and 7 constitute another group, stimulating a low rate of firing from neuron A, producing little reduction in the amount eaten in the no-choice bioassay and having no significant longer-term antifeedant activity, as seen in the results of the dual-choice GFD bioassay. The two groups are composed principally of chalcones in the one case and flavanones in the other. It may be that these two groups of compounds stimulate different receptor sites on the same neuron or the same sites with differing effectiveness.

In *S. littoralis*, the fact that the total neural response from the medial styloconic sensillum decreased as the amount eaten decreased is difficult to explain. However, when the neural input is examined in more detail, the response can be explained in terms of the concept of antifeedant compounds stimulating specific neurons (see review by Schoonhoven, 1986). Previous experiments with *S. littoralis* have shown that when a test solution contains both an antifeedant, such as azadirachtin, and a phagostimulant, such as sucrose, dissolved in sodium chloride, the response from the medial sensillum will be lower than that obtained with either the antifeedant or phagostimulant alone (Simmonds and Blaney, 1984). In that particular case, an interaction appeared to occur between neuron

A, responding to the deterrent, and neuron C, responding to the phagostimulant. Whether that interaction occurred between the receptor sites and the compounds or between neurons is not known. The effect seen in the present experiments is likely to be of a different nature since the compounds were dissolved in sodium chloride solution only, and no phagostimulant such as sucrose was present. Nevertheless, a more complex variant of the general theme of a specific deterrent neuron may apply with *S. littoralis*, with two possible modes of action being involved.

First, the decrease in firing with decreased palatability involves a decrease in the firing of both neuron A and neuron C. Compounds 2, 3, 11, 12, 13, and 17 stimulated predominantly neuron C, and compounds 3, 12, 13, and 17 yielded relatively high rates of firing from this neuron, whereas compounds 1, 5, 6, and 14 stimulated predominantly neuron A with relatively low rates of firing (Table 4). In all cases, neuron D was also firing, but at a lower rate than occurred with the control solution, sodium chloride. In general, as palatability decreased, the rate of firing of neuron A increased as a proportion of the total firing. Thus the lack of palatability of some of the compounds is signaled by the response of neuron A. The importance of neuron A in the gustatory code for antifeedant activity has been shown previously for *S. littoralis* with the clerodane diterpenoid compounds (Blaney et al., 1988) and the alkaloidal glycosidase inhibitors (Simmonds et al., 1989).

Second, it is possible that the different levels of response seen in Figure 4 actually derive from the same neuron, rather than two neurons. However, we have been very aware of this possibility and have studied spike height histograms, spike shape profiles, and temporal sequences meticulously in identifying spikes from neurons A, C, and D (Frazier and Hanson, 1986; Blaney et al., 1987, 1988).

Given the fact that we are dealing with a group of structurally similar compounds, it is interesting that, with *S. littoralis*, they appear to be able to stimulate receptors on at least two neurons, and a slight change in the ratio with which they stimulate these neurons correlates with their ability to affect feeding behavior.

The level at which the receptors on each neuron respond to a specific compound may be related to how the compounds engage in stimulus–receptor–ionophore interactions. These interactions would result in different levels of response specific to the receptor sites on the different neurons. The present results suggest that there are at least two different receptor types involved, each having a different structure–function type of response; in *S. exempta* they are situated on one neuron, whereas in *S. littoralis* they are situated on different neurons. However, of the compounds studied here, no aspects of their molecular structure known to us allow us to identify which receptor they would stimulate without exceptions.

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ISOLATION AND IDENTIFICATION OF VOLATILE KAIROMONE THAT AFFECTS ACARINE PREDATOR–PREY INTERACTIONS

Involvement of Host Plant in Its Production

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Abstract—A volatile kairomone emitted from lima bean plants (*Phaseolus lunatus*) infested with the spider mite *Tetranychus urticae*, was collected on Tenax-TA and analyzed with GC-MS. Two components were identified as the methylene monoterpene (3*E*)-4,8-dimethyl-1,3,7-nonatriene and the methylene sesquiterpene (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, respectively, after purification by preparative GC on a megabore column and recording of UV, IR, and [^{1}H]NMR spectra. The response of two species of predatory mites towards the identified chemicals was tested in a Y-tube olfactometer. Four of the compounds tested, linalool (3,7-dimethyl-1,6-octadien-3-ol), (*E*)-β-ocimene [(3*E*)-3,7-dimethyl-1,3,6-octatriene], (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate attracted females of *Phytoseiulus persimilis*. Linalool and methyl salicylate attracted females of *Amblyseius potentillae*. The response of *A. potentillae* to these two kairomone components was affected by the rearing diet of the predators in the same way as was reported for the response to the natural kairomone blend: when reared on a carotenoid-deficient diet, the predators responded to the volatile kairomone of *T. urticae*, but when reared on a carotenoid-containing diet they did not. The identified kairomone components are all known from the plant kingdom. They are not known to be produced by animals de novo.

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In addition to biological evidence, this chemical evidence suggests that the plant is involved in production of the kairomone. Based on the present study and literature data on the response of *T. urticae* to infochemicals, it is concluded that the kairomone component linalool is also a component of a volatile spider-mite dispersing pheromone.

Key Words—Kairomone, spider mites, predatory mites, lima bean leaves, tritrophic system, methylene monoterpene, methylene sesquiterpene, rearing diet, carotene, identification.

INTRODUCTION

Since the first report on volatile kairomones (sensu Dicke and Sabelis, 1988) in interactions between phytophagous mites and predatory mites (Sabelis and Van de Baan, 1983), a lot of biological information has been obtained on many aspects of these acarine allelochemicals. Volatile kairomones have been reported from 13 acarine prey–predator interactions (Sabelis and Van de Baan, 1983; Sabelis and Dicke, 1985; Dicke and Groeneveld, 1986; Dicke et al., 1986, 1988; Dong and Chant, 1986; Dicke, 1988). Studies have concentrated on the effect of predator condition on the response (Sabelis and Van de Baan, 1983; Dicke and Groeneveld, 1986; Dicke et al., 1986; Dong and Chant, 1986; Dicke, 1988), the type of behavior elicited in the predator by the kairomone (Sabelis et al., 1984a), kairomone preference (Dicke and Groeneveld, 1986; Dicke, 1988), and extraction of the compounds from leaves with methylene chloride and study of their origin (Sabelis et al., 1984b).

As a result of the increased biological knowledge on volatile kairomones in spider mite—predatory mite interactions, questions have arisen that can only be answered by identification of the chemicals involved:

1. Do different predator species, which all respond to the same prey species, use the same chemicals as a source of information? The phytoseiid species *Phytoseiulus persimilis* Athias-Henriot, *Typhlodromus occidentalis* (Nesbitt), *Amblyseius potentillae* (Garman), and *Typhlodromus pyri* Scheuten, all respond to a volatile kairomone of the two-spotted spider mite, *Tetranychus urticae* Koch (Sabelis and Van de Baan, 1983; Dicke et al., 1986; Dicke, 1988). *Tetranychus urticae* is a valuable prey species to the first two predator species, but the latter two are severely hindered by the dense web structure of this prey, which reduces predator reproductive success (Sabelis, 1981; Overmeer, 1981; McMurtry et al., 1970). This difference in profitability is reflected in the response of the predators to a volatile kairomone of *T. urticae*. *Phytoseiulus persimilis* and *T. occidentalis* respond to the kairomone when satiated or starved for 24 hr, respectively (Sabelis and Van de Baan, 1983), but severe quantitative or qualitative food deficiencies are needed to demonstrate the response of *A.*

potentillae and *T. pyri* to the volatile kairomone of the two-spotted spider mite (Dicke et al., 1986; Dicke, 1988; Dicke, unpublished data). It is interesting to know whether the different effects of *T. urticae* on reproductive success of these four phytoseiid species are also reflected in differences in the chemical information used by the predators.

2. What is the function of the volatile kairomone to the prey mite itself? The predators may cue in on any spider mite-related chemical, but as a result of natural selection, only a response to a chemical that is indispensable or inevitable to the prey mite will last long on an evolutionary time scale. From leaves infested with *T. urticae*, a volatile spider-mite dispersing pheromone is emitted, the biological characteristics of which suggest that it may be (partly) identical to the kairomone (Dicke, 1986). However, only identification of the chemicals involved in the intraspecific and interspecific interactions may demonstrate this.

3. What is the origin of the kairomone? An interaction of the host plant and the spider mite is a prerequisite for demonstration of a response by *P. persimilis* (Sabelis et al., 1984a; Dicke, unpublished data). The kairomone in the current tritrophic system is present on previously infested leaves from which all spider mites and visible spider-mite products have been removed (Sabelis et al., 1984a). This indicates that the kairomone may have been produced by the plant and/or by the spider mites that deposited it on the plant. Chemical identification of the kairomone may assist in revealing the origin of the kairomone and the role of the plant and the spider mite in its production.

Here we report on the isolation, by collection of airborne chemicals, and identification of components of the volatile *T. urticae* kairomone.

METHODS AND MATERIALS

Mites. The two-spotted spider mite (*T. urticae*) was reared on lima bean plants (*Phaseolus lunatus* L.) at 20–30°C, 50–70% relative humidity under continuous fluorescent light that was added to the natural daylight regime.

The predatory mite *P. persimilis* was obtained from Koppert BV (Berkel en Rodenrijs, The Netherlands) six years ago. It was reared at ca. 20°C on lima bean leaves infested with *T. urticae*, either on wet cotton wool or on clay flower pots that were put upside down in a water basin.

The predator *A. potentillae* was reared on either of two diets: *Vicia faba* (broad bean) pollen or *T. urticae*. In both cases, the food was provided on plastic arenas (McMurtry and Scriven, 1965; Overmeer, 1985). The predators of these two cultures will be indicated by the suffixes (Vf) and (Tu) respectively. For additional information on rearing history, see Dicke et al. (1989).

Isolation of Kairomone for Analytical Purposes. Twenty lima bean plants, with two to three leaves per plant, were cut and immediately placed in a 5-liter

glass jar with 1 cm of freshly distilled water at the bottom. Care was taken not to damage the plants except for cutting the stem. Three plant treatments were used: clean, infested with spider mites, or damaged mechanically by rubbing the leaves with carborundum on a wet cotton wool pad. A Pyrex glass tube (161 × 6.4 mm OD; ID 3 mm) with 90 mg of Tenax-TA in the center, held in place by two plugs of silanized glass wool, was connected to the outlet of the jar, and a charcoal filter to the inlet. Air was sucked through the system with a membrane pump at ca. 15 ml/min, for 1, 3, 5, 15, or 60 min.

The collected volatiles were released from the adsorbent by heating in a Thermodesorption Cold Trap Unit (Chrompack, Middelburg, The Netherlands) at 250°C for 3 min, with an N_2 flow of 20 ml/min. The desorbed compounds were collected in a cold trap (SIL5CB-coated, fused silica capillary) at −100°C. Flash heating of the cold trap provides sharp injection of the compounds into the capillary column of the gas chromatograph to which the cold trap is connected.

This desorption procedure is inapplicable when thermolabile compounds are present, but that was not the case here: no qualitative differences were observed between the chemicals identified with thermodesorption and those identified after solvent extraction of Tenax on which volatiles were collected from plants infested with *T. urticae*. Thermodesorption was used here because it provides a more sensitive analysis method: all desorbed volatiles are injected into the GC at the same time.

Isolation of Kairomone for Preparative Purposes. Two compounds that could not be identified by analytical GC and GC-MS were collected in larger amounts and purified by preparative GC for subsequent analysis by GC-IR and NMR.

Freshly picked infested lima bean leaves (500–600 g) were put in a stainless-steel cylinder (24.7 cm ID, 40.4 cm high) and flushed with 2.5 ml purified air per second overnight. The air was first pumped through glass tubes containing KOH pellets, mixed molecular sieves 5A and 13X, activated charcoal, and Tenax-TA before entering the cylinder at the center top. The kairomone-containing air left the cylinder at the bottom through a perforated stainless-steel ring near the wall of the cylinder. Via a Teflon tube, the air was led through a glass trap (4.8 mm ID) containing Tenax-TA (ca. 200 mg). The Tenax was cleaned before use by overnight extraction in a Soxhlet apparatus with MeOH, EtOAc, and hexane, respectively. The Tenax with the collected volatiles was extracted with 4 ml pentane–acetone (4 : 1) for 30 min in a micro-Soxhlet on a glass frit. The extract was poured in a conical 5-ml flask and an insulated 25-cm Vigreux column was put on top of the flask. The solution was concentrated to 0.3 ml in a water bath of ca. 42°C. The above procedure was repeated nine times. All the concentrated extracts were combined and concentrated to 0.6 ml. In ca. 20 injections of 25 μl each, the compounds were separated on a megabore

column. The two compounds of interest were trapped in two glass traps cooled to −70°C (the FID was disconnected during trapping). When the traps were not connected to the GC, they were closed with teflon tubing.

Instruments Used. For analytical GC a Varian 3500 gas chromatograph equipped with a 30-m DB-17 (J&W) column was used; split ratio 1 : 100, carrier gas nitrogen; temperature program 60°C (0 min hold) to 210°C (0 min hold) with 2.5°C/min; FID. For preparative purposes a Carlo Erba gas chromatograph equipped with a 30-m DB-1 (J&W) column was used; 0.53 mm ID; 3.0 μm film thickness; no split; carrier gas nitrogen; temperature program 60°C (0 min hold) to 150°C at 5°C/min, then to 210°C at 10°C/min, 10 min hold.

All volatiles were analyzed by GC-MS using a Chrompack SIL19CB column (25 m × 0.25 mm ID). The column temperature was held at ambient for 2 min, then at 40°C for 4 min, and then programmed to 280°C at a rate of 4°C/min. Electron impact ionization was carried out at 70 eV on a VG MM7070F mass spectrometer (resolving power 1000).

The 300-MHz [^{1}H]NMR spectra were recorded in $CDCl_3$ on a Bruker CXP300. The $C\underline{H}Cl_3$ signal was used as an internal reference (7.26 ppm). Assignments were confirmed by homo-nuclear decoupling experiments.

For GC-IR, a Carlo-Erba gas chromatograph equipped with a 30-m DB-1 (J&W) column (film thickness 1 μm, split 1 : 5) connected with a Bruker IFS-85 FT-IR detector was used.

For HPLC, a Gilson 303 pump and a Gilson 116 UV detector were used in combination with a Rainin C18 column, 10 cm × 4.6 mm, 3 μm particle size. Solvent: MeOH–H_2O (9 : 1); flow rate 1 ml/min; detection UV 220 nm.

Synthesis. To synthesize (3*E*)-4,8-dimethyl-1,3,7-nonatriene, geraniol was oxidized with CrO_3 pyridine to geranial, which was converted to the desired product with the Wittig reagent methyltriphenylphosphonium iodide.

To synthesize (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (2*E*, 7*E*)-3,7,11-trimethyl-2,7,10-dodecatriene-1-ol (Aldrich) was oxidized with CrO_3 in pyridine to the corresponding aldehyde. This aldehyde was submitted to a Wittig reaction using CH_2I_2, Zn, and $TiCl_4$, yielding the desired product.

Solvents. All solvents used were of analytical or HPLC quality.

Separation of (*Z*)- and (*E*)-β-Ocimene. β-Ocimene (3,7-dimethyl-1,3,6-octatriene) could only be obtained as a mixture of the *Z* and *E* isomers. This mixture was separated by preparative GC on a 9.8% Carbowax on Chromosorb W-HP 100–120 column (200 × 0.2 cm), with a TCD detector and a column temperature of 80°C. The desired isomer was collected in a capillary tube which was cooled in Dry Ice. The ratio of the two isomers in the collected fractions was determined on the same column.

Bioassay. To determine whether extracts or synthetic chemicals had kairomonal activity, a Y-tube olfactometer was used. A glass Y-shaped tube held a Y-shaped iron wire in the center. At the end of both arms of the olfactometer,

filter paper (15 cm^2) with test or control solutions was placed in PVC cages. Air was sucked out at the base of the tube and led to the outside of the climate room in which the olfactometer was positioned. The air speed in both arms of the olfactometer was measured with a hot wire anemometer and standardized at 0.7 ± 0.1 m/sec by adding dry cotton wool in the PVC cages and/or changing the total airflow speed with a valve. Female predators were placed on the wire in the basal tube and observed individually. When the predator walked upwind and reached the far end of one of the arms, the experiment was terminated. A sign test was used to analyze the results ($\alpha = 0.05$). The experiments were performed at $25 \pm 1°C$ and $60 \pm 10\%$ relative humidity.

Only female predators were tested in the olfactometer. In the case of *P. persimilis*, they were well-fed, but unless stated otherwise, *A. potentillae* females were tested after a 20-hr period of starvation in a plastic tube at 25°C.

The chemicals were dissolved in dichloromethane [(*Z*/*E*)-β-ocimene, linalool (3,7-dimethyl-1,6-octadien-3-ol) and methyl salicylate], paraffin oil [1-octen-3-ol, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, 4,8-dimethyl 1,3,7-nonatriene] or hexane [(*Z*/*E*)-β-ocimene]. Aliquots of 0.1 ml of diluted chemicals were poured onto a piece of filter paper outside the climate room and transferred to the olfactometer immediately. A filter paper with 0.1 ml solvent served as control. In these tests, new filter papers were prepared for each predator tested. All chemicals were obtained from Aldrich except for (*Z*)-3-hexen-1-yl acetate, which was a Roth product; β-ocimene, which was obtained as a mixture of isomers from International Flavor and Fragrances Industries or from Dr. J. H. Tumlinson (USDA, Gainesville, Florida) and (3*E*)-4,8-dimethyl-1,3,7-nonatriene isomers and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, which were synthesized (see above). All chemicals, except (*Z*/*E*)-β-ocimene (see Results) and the methylene terpenes, were ≥ 98% pure. The methylene terpenes were ca. 90% pure, as a result of rapidly occurring racemization.

RESULTS

Collection of Kairomone and Identification. The suitability of Tenax-TA to collect the volatile kairomone of *T. urticae* was demonstrated in the olfactometer with satiated females of *P. persimilis*: 31 of 49 predators chose the arm with solvent extract of Tenax on which volatiles of spider mite-infested plants had been collected ($P = 0.04$). By GC-MS analysis of the mixture of volatiles collected from spider mite-infested plants on Tenax-TA, many components could be distinguished (Table 1, Figure 1). Only three of these (2-butanone, 2-hexanal 1-octen-3-ol) have been found in samples collected from clean intact bean plants (Table 1). Some others, such as the general green leaf volatiles (*Z*)-3-hexen-1-

TABLE 1. VOLATILES COLLECTED FROM INTACT, ARTIFICIALLY DAMAGED, AND *T. urticae*-INFESTED LIMA BEAN PLANTS AND THEIR FUNCTION AS KAIROMONES FOR PHYTOSEIID MITES *P. persimilis* OR *Amblyseius potentillae* REARED ON *V. faba* POLLEN (Vf) OR *T. urticae* (Tu) OR AS DISPERSING PHEROMONE FOR SPIDER MITE *T. urticae*[a]

		Identified in			Component of			
					Kairomone of			
						A. potentillae		Dispersing
Peak number	*Tentative identification*	*T. urticae* infested plants	Artificially damaged plants	Undamaged plants	*P. persimilis*	(Vf)	(Tu)	pheromone of *T. urticae*
1	2-Butanone	+	+	+				
2	2-Methyl-propan-1-ol	+	−	−				
3	1-Butanol	+	−	−				
4	3-Pentanone	+	−	−				
5	1-Penten-3-ol	+	−	−				
6	Hexanal	−	+	+				
7	Unidentified (MW = 98)	+	+	−				
8	2-Hexenal	+	+	+				
9	(Z)-3-Hexen-1-ol	+	+	−	−	−	−	
10	(Z)-3-Hexen-1-yl acetate	+	+	−	−	−	−	
11	1-Octen-3-ol	+	+	+	−	−	−	
12	(*E*)-β-Ocimene	+	−	−	+	?[b]	?[b]	
13	(3*E*)-4,8-Dimethyl-1,3,7-nonatriene	+	−	−	+			
14	Linalool	+	−	−	+	+	−	+
15	(Z)-3-Hexen-1-yl butyrate	+	+	−				
16	Methyl salicylate	+	−	−	+	+	−	−
17	(3*E*,7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	+	−	−				

[a] *P. persimilis* were tested as satiated females, *A. potentillae* as females that were starved for 24 hr at 25°C. Data on *T. urticae* are based on the data of Dabrowski and Rodriguez (1971), Rodriguez et al. (1976) and Dicke (1986).
[b] Uncertain because pure isomer was not tested.

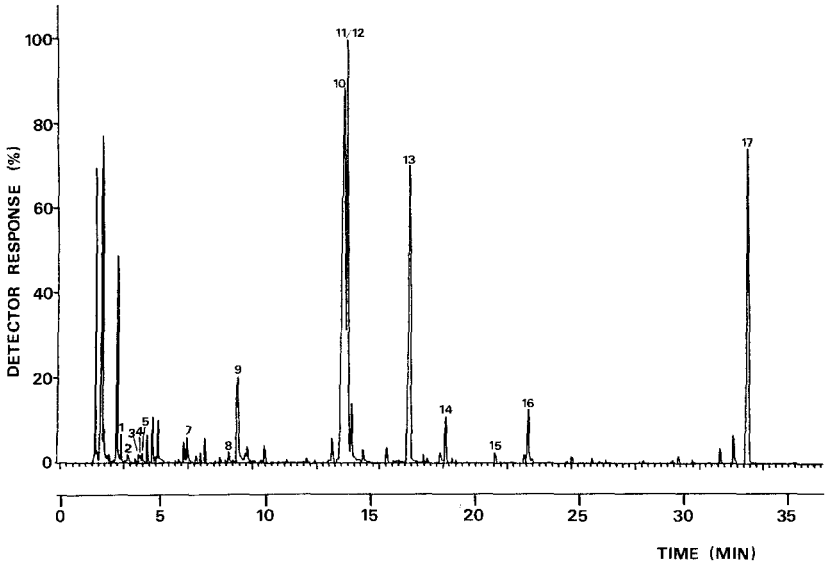

FIG. 1. Gas chromatogram of volatiles from spider mite-infested bean plants collected on Tenax-TA. Peak numbers correspond to numbers given in Table 1. Most unlabeled peaks are artifactual peaks.

ol and (Z)-3-hexen-1-yl acetate are expected to be emitted by lima bean plants, but if so, the quantities were very small compared to the amounts of these compounds that were obtained from spider mite-infested bean plants. An analysis of the volatiles released by artificially damaged plants revealed several compounds that are also emitted by spider mite-infested plants (Table 1). Thus, emission of these compounds from spider mite-infested plants presumably is the result of mechanical damage of the plant. The other volatiles emitted from *T. urticae*-infested bean plants are caused by other spider-mite activities or are emitted from the spider mites themselves.

Two major components of the volatile kairomone could not be readily identified. Their mass spectra showed the molecular ion at *m/z* 150 and *m/z* 218, respectively, with similar fragmentation patterns (base peak for both at *m/z* 69). The difference between the two compounds was most likely one isoprene unit. Initially they were thought to be the furanoterpenes perillene and dendrolasin because of the almost identical mass spectra. However, a sample of a ginger oil, which was investigated at the same time, contained both perillene and the unknown compound. As it was not possible to deduce the correct structures from MS alone, we tried to isolate the two compounds in larger quantities and to record [^{1}H]NMR and IR spectra.

Initial attempts to separate and purify these two compounds by means of GC on a packed column failed. However, no separation could be obtained. The reason for this could be the low sensitivity of the TCD or the low inertness (leading to excessive tailing or decomposition of the compounds involved) of the column used. A separation by reverse-phase-HPLC was successful, but during isolation of the very small quantities of apolar pure compounds from the aqueous solvents, too many impurities were introduced for recording of high-quality NMR spectra. Also, evaporation of the solvent inevitably resulted in loss of the volatile compounds. Separation and purification of the two compounds was successful by preparative capillary GC on a megabore column, a relatively new technique. This technique combines good inertness with high separation power and a relatively high loadability. [^{1}H]NMR spectra showed that the resulting samples consisted of pure compounds. Altogether an estimated 300 μg (mol wt = 150) and 200 μg (mol wt = 218) were collected. The IR spectra, recorded immediately before NMR analysis, most closely resembled the IR spectra of monoterpene hydrocarbons. No evidence for the presence of any oxygen-containing functional group could be found. The most likely molecular formulas were thus $C_{11}H_{18}$ and $C_{16}H_{26}$ instead of $C_{10}H_{14}O$ (perillene) and $C_{15}H_{22}O$ (dendrolasin). From the NMR data, structure **1** (Figure 2) or its *Z* isomer followed for the lower molecular weight compound. Especially the signal at 6.58 ppm with coupling constants of 17, 10.5, and 10.5 Hz (corresponding with H-2) was most helpful in elucidating the structure. As it was not possible to determine whether the compound possessed the *E* or *Z* configuration, both were synthesized. By comparison of the spectral data and retention times of the isolated compound and the synthesized isomers, it was clear that the isolated compound had the *E* configuration. The higher-molecular-

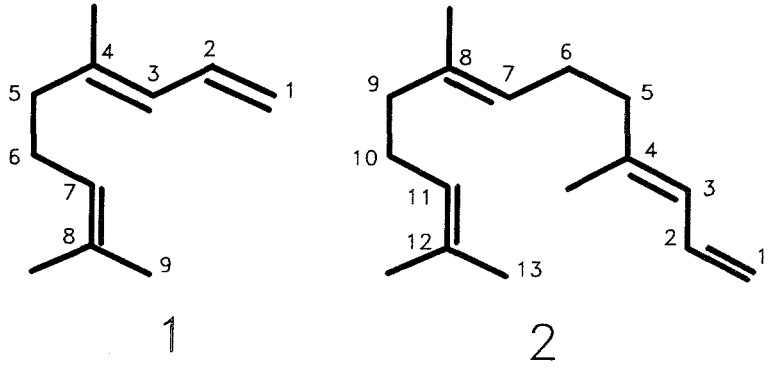

FIG. 2. Structures of the methylene terpenes (3*E*)-4,8-dimethyl-1,3,7-nonatriene (**1**) and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (**2**).

weight compound was now assigned structure **2** (Figure 2). The configuration around the 3,4 double bond is the same as in **1**, because of the identical chemical shifts and coupling constants. The configuration around the 7,8 double bond was deduced to be *E* from the chemical shift of the 7-methyl group at 1.61 ppm. In the case of the opposite configuration, the 7-methyl group would have appeared around 1.68 ppm. The structure of compound **2** was also confirmed by synthesis.

Bioassay of Identified Components. The effect on the predatory mite *P. persimilis* of those identified chemicals that were available was tested in the Y-tube olfactometer. Figure 3 shows that linalool is attractive to *P. persimilis* females at a wide dose range, whereas methyl salicylate proved to be attractive only when offered at an amount of 20 μg. The dose range was based on the finding that the amounts of each compound recovered from 20 plants during 60 min of sampling were in the range of one to several tens of micrograms. (*Z*)-3-Hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, and 1-octen-3-ol did not attract the predators. These latter compounds had been identified in samples obtained from both artificially damaged and spider mite-infested plants.

The data for ocimene are presented in Figure 4. β-Ocimene was obtained as mixtures of the *E* and *Z* isomers at ratios of 90:10 and 70:30. Gas chromatography on 3% OV-17 showed that some minor compounds were also present. When tested in the olfactometer, only the 90:10 (*E*, *Z*)-β-ocimene mixture proved attractive to *P. persimilis*, either when 20, 2, or 0.2 μg were offered. When the dose offered was only 0.02 μg, no response was observed. The difference in response of *P. persimilis* to the two β-ocimene samples could be

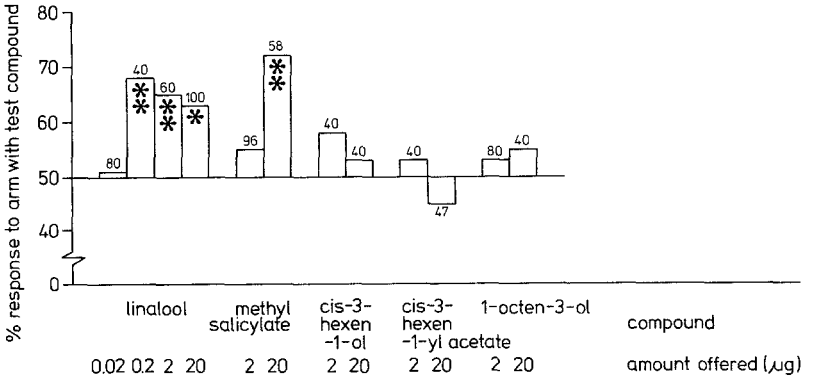

FIG. 3. Choice of *P. persimilis* females in Y-tube olfactometer to various doses of chemicals identified after collection of volatiles emitted from *T. urticae*-infested lima bean plants. Numbers under and above columns indicate the number of predators tested. **$0.001 < P < 0.01$; *$0.01 < P < 0.05$.

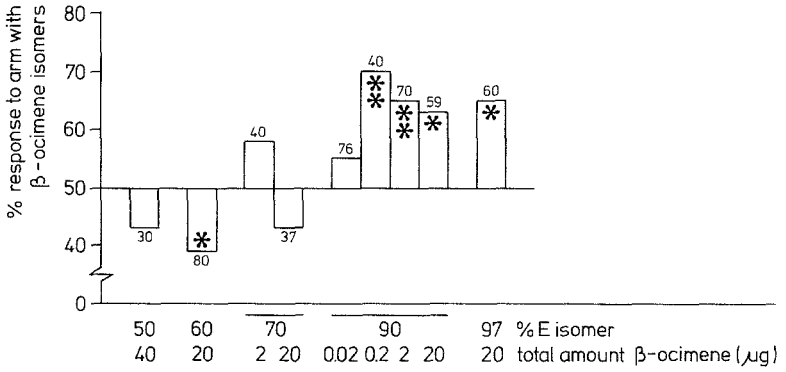

FIG. 4. Choice of *P. persimilis* females in Y-tube olfactometer to β-ocimene, offered at various *E*/*Z* ratios and various doses. Numbers under and above columns indicate the number of predators tested. For explanation of asterisks, see Figure 3.

caused by the impurities or by the difference in *E*/*Z* ratio. To investigate this, *E*/*Z* ratios of 50:50 and 60:40 were prepared from the 90:10 sample and a 97:3 *E*/*Z* ratio from the 70:30 sample. The 97:3 sample was attractive, but the 50:50 sample was not. The 60:40 *E*/*Z* ratio was even repellent. These data show that (*Z*)-β-ocimene reduced the attractivity of (*E*)-β-ocimene to *P. persimilis*.

Experiments with the methylene terpenes (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (3*E*, 7*E*)-1,4,8-trimethyl,1,3,7,11-tridecatetraene show that 20 μg of the former compound is also attractive to *P. persimilis*, whereas its *Z* isomer is not (Figure 5). No response of *P. persimilis* towards (3*E*, 7*E*)-1,4,8-trimethyl-1,3,7,11-tridecatetraene was recorded (Figure 5).

Amblyseius potentillae responded to the natural kairomone blend when reared on a carotenoid-deficient diet such as *V. faba* pollen, but not when reared on the carotenoid-containing spider mite *T. urticae* (Dicke et al., 1986). Predators that differed in qualitative feeding history were offered the identified chemicals. The results (Figure 6) show that when amounts of 20 μg were offered, no chemical was attractive to *A. potentillae* (Tu). However, when the predator was reared on *V. faba* pollen, methyl salicylate and linalool were attractive, which resembles the data obtained for *P. persimilis*. However, in contrast to *P. persimilis*, *A. potentillae* (Vf) did not respond to 90:10 *E*/*Z* β-ocimene. Either (*E*)-β-ocimene is not attractive or the presence of 10% of the *Z* isomer or the other impurities disturbed the response of *A. potentillae* to the *E* isomer.

The response of *A. potentillae* to linalool was studied in more detail (Figure 7). Three different concentrations were tested: 20, 2, and 0.2 μg. When reared on *V. faba* pollen, all three concentrations attracted predators that were

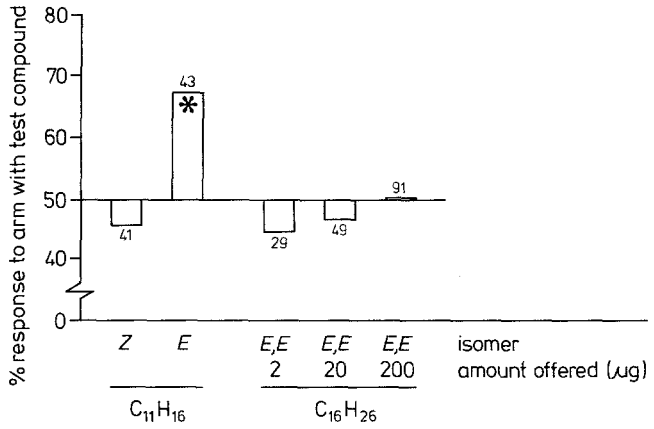

FIG. 5. Choice of *P. persimilis* females in Y-tube olfactometer to 4,8-dimethyl-1,3,7-nonatriene isomers and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Numbers under and above columns indicate the number of predators tested. For explanation of asterisks, see Figure 3.

starved for 20 hr. When satiated, these predators also responded to 2 µg linalool. A response to the volatile *T. urticae* kairomone in well-fed *A. potentillae* has only been observed when the predators were reared on *V. faba* pollen (Dicke, unpublished data). In contrast to the data for *A. potentillae* (Vf), amounts of

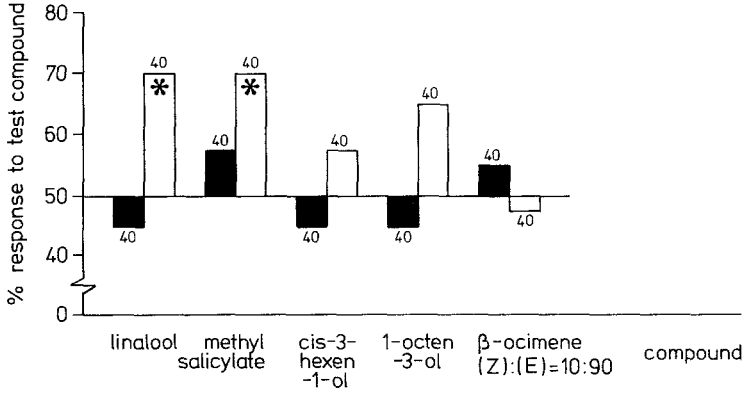

FIG. 6. Choice of starved female *A. potentillae* (Tu) (black bars) and *A. potentillae* (Vf) (white bars) in Y-tube olfactometer to synthetic chemicals (dose: 20 µg), identified after collection of volatiles from *T. urticae*-infested lima bean plants. For explanation of asterisks, see Figure 3. Numbers under and above columns indicate the number of predators tested.

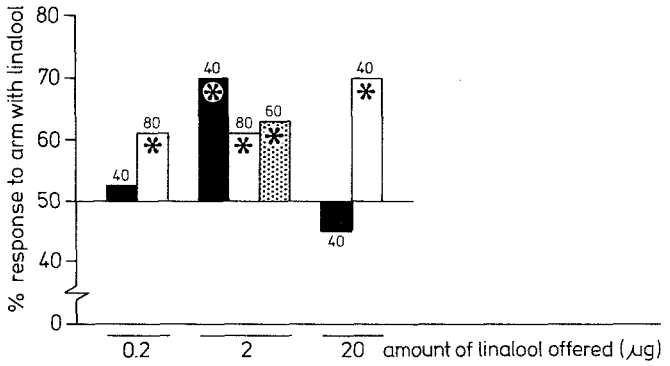

FIG. 7. Choice of starved female *A. potentillae* (Tu) (black bars) and starved and satiated *A. potentillae* (Vf) (white bars: starved; shaded bar: satiated) in Y-tube olfactometer to linalool at various doses. For explanation of asterisks, see Figure 3. Numbers under and above columns indicate the number of predators tested.

20 and 0.2 μg linalool were not attractive to *A. potentillae* (Tu) that were starved for 20 hr, but when 2 μg linalool was offered, a significant response was observed in these predators. The data for the responses of both predator species are summarized in Table 1.

DISCUSSION

Damage inflicted on lima bean plants by rubbing the leaves with carborundum or by infestation with spider mites caused emission of many volatiles. However, a clear difference was observed between the volatile mixture emitted from plants damaged in either way. Most of the major volatiles emitted from clean lima bean plants, plants damaged mechanically, or plants infested with *T. urticae* were identified. The amounts of volatiles emitted from spider mite-infested plants were large enough to allow short sampling times in our investigation. However, minor compounds may have been missed.

Shortly after our identification of the two methylene terpenes (Figure 2), Maurer et al. (1986) reported the isolation of these compounds from *Elettaria cardamomum* (cardamom) oil, which, like ginger, is a species from the Zingiberaceae family. The spectral data reported by Maurer et al. (1986) are identical to the data recorded by us. In 1987, independent of our work, the same compounds were reported by Kaiser (1987) from flowers of many night-scented plant species of different families that are pollinated by moths.

The odor differences observed between plants that are in different condition, will allow many organisms to distinguish between them. This will be discussed according to the three questions posed in the Introduction.

1. Do different predator species use similar chemicals as volatile *T. urticae* kairomone? Four compounds elicited a significantly attractive response from at least one of the predator populations tested: these compounds are the terpenes linalool and (*E*)-β-ocimene, the methylene terpene (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate.

All four compounds attracted *P. persimilis* females although methyl salicylate only elicited this response when offered at a dose of 20 μg. The effect of linalool and methyl salicylate on *A. potentillae* (Vf) is similar to that on *P. persimilis*. With respect to (*E*)-β-ocimene, no definitive conclusion can yet be made, because it is not known whether the presence of 10% *Z* isomer affected the behavior of *A. potentillae* (Vf) towards (*E*)-β-ocimene.

With regard to the kairomone components linalool and methyl salicylate, the data obtained for *A. potentillae* reared on either of the two diets are in agreement with those of Dicke et al. (1986) and Dicke (in preparation) on the response of *A. potentillae* to the volatiles emitted by *T. urticae*-infested plants. The only exception is the response of *A. potentillae* (Tu) to 2 μg linalool. This may indicate that *A. potentillae* (Tu) are sensitive to this component of the volatile *T. urticae* kairomone, although much less than *A. potentillae* (Vf), which respond much more consistently to linalool.

Our observations provide no reason to suppose that different predator species use different volatiles emitted from *T. urticae*-infested plants as kairomone. Investigations with other phytoseiid species, such as *T. occidentalis* or *T. pyri* are needed.

2. Do kairomone components function as dispersing pheromone for spider mites? The spider mite *T. urticae* also distinguishes between undamaged and *T. urticae*-damaged lima bean plants. Clean leaves emit a volatile kairomone that attracts spider-mite females. After infestation with *T. urticae*, the leaves emit a dispersing pheromone in addition. At a high ratio of dispersing pheromone to plant kairomone, the spider mites are repelled (Dicke, 1986).

It is interesting to investigate whether the chemicals that act as a kairomone to predatory mites act as a spider-mite dispersing pheromone. Some indications for this can be found in a study on host plant–spider mite interaction. Dabrowski and Rodriguez (1971) studied the response of *T. urticae* females to essential oil components of strawberry foliage. In a static air olfactometer, linalool repelled the mites at several concentrations. Methyl salicylate was indifferent, whereas (*E*)-β-ocimene or (3*E*)-4,8-dimethyl-1,3,7-nonatriene were not included in their study. In a later study by Rodriguez et al. (1976), mixtures of compounds were used. A mixture resembling strawberry essential oil attracted *T. urticae* females. An increase in the proportion of nonanal, one of the components, reduced the attractive effect. However, an increase in the proportion of methyl salicylate had no effect. Unfortunately, the effect of a change in the proportion of linalool was not studied.

In the context of the present study and the one by Dicke (1986), the data of Dabrowski and Rodriguez (1971) indicate that linalool is not only a component of the *T. urticae* kairomone that attracts predatory mites, but also of the *T. urticae* dispersing pheromone. This supports the hypothesis that the dispersing pheromone and the spider-mite kairomone have components in common (Dicke, 1986). There is no evidence that the kairomone component methyl salicylate is a component of the dispersing pheromone (Rodriguez et al., 1976). These data on the identity of components of the *T. urticae* dispersing pheromone are incorporated into Table 1. It will be interesting to investigate the effect of (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene on *T. urticae* females to know whether these kairomone components are also a component of the dispersing pheromone.

3. What is the origin of the volatile kairomone? The above considerations raise the question of the origin of the allelochemicals that function in plant–spider mite–predatory mite interactions. The chemicals presumably are of plant origin, as can be inferred from the classes of compounds identified (e.g., Karrer, 1976; Maurer et al., 1986). However, it cannot be concluded yet that the plant has active control over the production or emission of the volatiles or that the plant offers a substrate for spider-mite enzymes that subsequently produce the kairomone components. Investigation of, e.g., site and moment of production and possible storage of precursors are needed for that. Chemical identification of kairomone components was an essential first step for this.

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MAMMALIAN PHEROMONES VIII[1]
Chemical Characterization of Preorbital Gland Secretion of Grey Duiker, *Sylvicapra grimmia* (Artiodactyla: Bovidae)

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Abstract—Using gas chromatography–mass spectrometry 33 constituents of the preorbital gland of the grey duiker, *Sylvicapra grimmia*, were identified as unbranched alkanes, 2-alkanones, alkanals, alkanoic acids, alkan-4-olides, as well as 3-methyl-3-buten-1-ol, (Z)-9-octadecenoic acid, benzyl cyanide, diethyl sulfoxide, 2-isobutyl-1,3-thiazole, 2-isobutyl-4,5-dihydro-1,3-thiazole, and 3,4-epoxy-2-dodecanone. Determination of the relative concentrations of these components in a limited number of secretion samples taken from males and females revealed that in these samples the two thiazole derivatives and the epoxy ketone were present in consistently and significantly higher concentrations in male than in female secretions. This suggests that they could act as sex recognition cues.

Key Words—*Sylvicapra grimmia*, Bovidae, semiochemicals, pheromones, exocrine secretion, preorbital secretions, thiazoles, epoxy ketone, mammalian gland secretion, antelope, grey duiker.

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INTRODUCTION

In many groups of mammals odoriferous substances that act as semiochemicals are produced by cutaneous glands. These glands are common in carnivores and rodents and in most ungulate groups, but are also found in many other orders (Schaffer, 1940). Volatile substances may be released into the atmosphere by diffusion directly from the glands, but in most cases that have been 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 several bovid species, such as the grysbok, *Raphicerus melanotis*, and the grey duiker, *Sylvicapra grimmia*, employ secretions from their preorbital glands for this purpose. These small antelope cause damage of some economic significance 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.

This research program was started with the chemical characterization of the preorbital gland secretion of the grysbok, *Rhaphicerus melanotis* (Burger et al., 1981). A total of 27 straight-chain compounds, which can be classified as alkanols, alkenols, alkadienols, alkanyl methanoates, alkenyl methanoates, and alkanoic acids, were identified in the secretion.

Although the ritual involved in marking objects with preorbital gland secretions undoubtedly plays an important part in the behavior of male grysbok, there is no experimental evidence to indicate whether the olfactory stimuli released from the deposited material serve as deterrents signalling territorial occupancy to other males (Novellie et al., 1984). It is, however, possible that olfactory cues play a more important part in the territorial behavior of more primitive species than in that of the grysbok. The grey duiker, *Sylvicapra grimmia*, a small cephalophine antelope, is considered by some authors to fall into this category (Estes, 1974) and was selected for investigation. In this paper the identification of the volatile organic constituents of the preorbital gland secretion of the grey duiker, and the results of the quantitative determination of these constituents in individual males and females, are reported.

METHODS AND MATERIALS

General. Dichloromethane (Merck, Residue Analysis Grade) was analyzed gas chromatographically and found to be pure enough for extraction purposes when used in small quantities. All Pyrex glassware used in the handling of biological material and extracts thereof was heated to 500°C in an annealing

oven to remove traces of organic material. Syringes, stainless-steel needles, etc., that were used to handle material, were cleaned by rinsing with the dichloromethane specified above.

Analytical Methods. Gas chromatographic analyses (GC) were carried out with a Carlo Erba 5300 (Mega) gas chromatograph equipped with a flame ionization detector, a Grob split–splitless injector and a 45-m Pyrex capillary column (ID 0.32) coated with a 1.0-μm film of PS-089-OH (Petrarch Systems, Bristol, Pennsylvania) which is a silanol-terminated (95%)–dimethyl–(5%)–diphenylsiloxane copolymer. The column was manufactured according to procedures developed by Blum (1985) for similar silanol-terminated phases. 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 260°C. The injector was normally used at 200°C. Samples were injected in the splitless mode and the split valve was opened after 1 min. The volatiles entering the column were thermally focused and subsequently analyzed using the following temperature program: 25°C (1 min), programmed at 25°C/min to 40°C (5 min) and programmed at 2°C/min to 240°C (hold).

GC data were stored and processed with a Hewlett-Packard XTRA CHROM II Data System using a Nelson Analytical 192-K interface. Electron-impact (EI) mass spectra were recorded at 70 eV on a Finnigan MAT 4500 GC-MS instrument, using the GC column and conditions described above. Chemical-ionization (CI) mass spectra were obtained with methane as reactant gas. High-resolution mass-spectral data were obtained with a Varian MAT 311A mass spectrometer and a Kratos DS90 data system. [^{1}H]- and [^{13}C]NMR spectra were recorded at 300 MHz and 75 MHz, respectively, on a Varian VXR-300 spectrometer using a probe temperature of 26°C.

Sample Preparation and Analysis. Preorbital gland secretion was collected individually from netted or culled, sexually mature male and female animals by scooping the exudate with Reacti-Vials from the row of preorbital pores through which it is released from the underlying preorbital gland. To avoid handling the very sensitive duikers, material was also collected from territorial marks, freshly deposited on precleaned aluminum foil. The organic volatiles were extracted by vigorously shaking each collected sample (0.4–1.0 g) with dichloromethane (0.5–1.2 ml) in a mechanical flask shaker for 6 hr, centrifuging the resulting emulsion for 2 hr at 3000 rpm, removing the dichloromethane extract from underneath the supernatant water and mucus layer with a 1000-μl syringe and transferring it to a clean Reacti-Vial. These extracts were further concentrated in an inert, purified (activated charcoal) nitrogen atmosphere for GC-MS analysis. For quantitative comparison of the secretions of individual grey duikers, these extracts were diluted with dichloromethane to approximately the original volume of the collected material. Thus, an extract was diluted to, for instance, 430 μl if 430 mg of material had initially been collected from an animal. The

extracts obtained by this procedure were used without further concentration to obtain quantitative gas chromatographic data.

To avoid the introduction of artifacts into the extracted organic material by the use of a solvent, the authenticity of the constituents identified in the secretion was verified by collecting secretion in glass capillaries that were introduced into the inlet of the gas chromatograph without any further sample preparation. This was achieved by using a slightly adapted version of the solventless injection technique described by Burger et al. (1985). A strand of glass wool was inserted very loosely into the first section (25 mm) of a glass capillary (80 mm × 1.0 mm ID). This section of the capillary was cooled (ca. −50°C) with a small copper heat sink, which had been precooled in liquid N_2. Secretion (ca. 10 μl) was carefully drawn into this part of the capillary, which was then sealed off at a point about 30 mm from its inlet end. The heat sink was removed and the sample-containing end of the capillary introduced to a depth of ca. 50 mm into the injector, maintained at a temperature of 200°C (splitless mode) of the gas chromatograph through an otherwise stoppered hole in the septum, while keeping the column at room temperature. After 1 min the capillary was replaced by a glass stopper, the split valve opened, and the temperature program started. In an alternative procedure, in which the sample is not subjected to excessively high temperatures, the crude secretion was introduced into the injector at a low temperature (60–100°C), whereafter the injector was ballistically heated to 200°C, thus allowing volatile material to evaporate and be transported onto the column at the lowest possible temperature. The final injector temperature was maintained for 2 min, whereafter the split valve was opened, the injector heater switched off and the capillary with involatile residue replaced by a glass stopper. In this procedure the temperature program was adapted to allow for an isothermal period of 2 min at ambient temperature, although using a shorter isothermal period did not materially affect the efficiency of the GC separation.

Synthetic Reference Compounds. Synthetic samples of some of the compounds identified in the preorbital gland secretion of the grey duiker were obtained from Merck (Darmstadt), Fluka (Buchs), and Aldrich (Milwaukee, Wisconsin). Compounds that are not commercially available were synthesized. Melting and boiling points are uncorrected.

Octadecan-4-olide was prepared from 1-hexadecene according to the method of Heiba et al. (1974). The reaction product was purified by distillation at 158°C/2 torr (yield 43%) and recrystallization to give the pure lactone as fine colorless needles, mp 48.5–49.5°C (EtOH); [^{13}C]NMR ($CDCl_3$): δ 177.24 (s, C-1), 28.87 (t, C-2), 28.03 (t, C-3), 81.06 (d, C-4), 35.62 (t, C-5), 25.25 (t, C-6), 29.71 (t, C-7), 29.70 (t), 29.68 (t), 29.68 (t), 29.64 (t), 29.55 (t), 29.49 (t), 29.38 (t), 29.37 (t) (C-8 to C-15), 31.95 (t, C-16), 22.71 (t, C-17), 14.12 (q, C-18).

Hexadecan-4-olide was prepared in an analogous manner from 1-tetrade-

cene. Distillation and crystallization from ethanol gave the γ-lactone as colorless crystals (yield 46%) bp 121°C/0.01 torr, mp 41–43°C; [^{13}C]NMR ($CDCl_3$): δ 177.24 (s, C-1), 28.87 (t, C-2), 28.04 (t, C-3), 81.06 (d, C-4), 35.63 (t, C-5), 25.26 (t, C-6), 29.68 (t, C-7), 29.65 (t), 29.65 (t), 29.55 (t), 29.49 (t), 29.37 (t), 29.37 (t)(C-8 to C-13), 31.94 (t, C-14), 22.71 (t, C-15), 14.12 (q, C-16).

3-Dodecen-2-one (Scheme 1 below, **3**) was prepared by a crossed aldol condensation of acetone and nonanal (**1**) followed by an acid catalyzed elimination of water from the resulting hydroxy ketone **2,** according to the method given by Tishchenko and Stanishevskii (1963) for the preparation of 3-nonen-2-one. Nonanal trimer (14.2 g) was depolymerized by stirring it with 6 M HCl (2 ml) for 2 hr, whereafter acetone was added to the reaction mixture and the resulting solution slowly added to a stirred solution of 2.5 M NaOH (10 ml) in acetone (16 ml) over a period of 2 hr at a temperature that did not exceed 12°C. After another hour at room temperature, the reaction mixture was diluted with ice (40 g), neutralized with 6 M HCl, and the acetone removed on a rotary evaporator. The organic material was extracted with ether and the combined extracts washed with saturated $NaHCO_3$ and saturated saline solution. The ether solution was dried and after filtration and evaporation of the solvent, the residue was distilled (123°C/0.001 torr) to give the condensation product (3.3 g), which was refluxed in benzene (13 ml) with *p*-toluenesulfonic acid (7 mg) in the presence of anhydrous Na_2SO_4 (1.3 g) for 1 hr. The drying agent was removed by filtration and the filtrate washed with saturated $NaHCO_3$, whereafter the benzene solution was dried, filtered, and the solvent removed under reduced pressure. Distillation of the resulting residue gave 3-dodecen-2-one (**3**) (1.97 g, 65%) as a colorless liquid, bp 82°C/0.01 torr; [^{1}H]NMR ($CDCl_3$): δ 6.808 (1H, H-4, dt, $J_{3,4}$ = 16.0, $J_{4,5}$ = 6.9), 6.070 (1H, H-3, dt, $J_{3,4}$ = 16.0, $J_{3,5}$ = 1.5), 2.236 (3H, H-1, s), 2.223 (2H, H-5, m), 1.469 (2H, H-6, m), 1.274 (10H, H-7 to H-11, CH_2 envelope), 0.883 (3H, H-12, t, $J_{11,12}$ = 6.7); [^{13}C]NMR ($CDCl_3$): δ 26.80 (q, C-1), 198.68 (s, C-2), 131.32 (d, C-3), 148.63 (d, C-4), 32.52 (t, C-5), 28.16 (t, C-6), 29.24 (t, C-7), 29.24 (t, C-8), 29.40 (t, C-9), 31.89 (t, C-10), 22.69 (t, C-11), 14.10 (q, C-12).

Dodecen-2-one-3,4-epoxide (Figure 1 below, 6294) was prepared according to the method of Wasson and House (1957). A solution of 3-dodecen-2-one (**3**) (1.9 g) in methanol (12 ml) was treated with H_2O_2 (35%, 2.4 ml) and 6 M NaOH added over a period of 55 min at 15°C. The reaction mixture was stirred at 20–25°C for a further hour, whereafter it was diluted with water (13 ml) and extracted with ether. The combined ether extracts were washed three times with water and once with potassium iodide solution (3%). The usual work-up procedure gave the epoxy ketone 6294 as a colorless oil (1.24 g, 61% yield), bp 80°C/0.07 torr; [^{1}H]NMR ($CDCl_3$): δ 3.176 (1H, H-3, d, $J_{3,4}$ = 2.0), 3.074 (1H, H-4, ddd, $J_{3,4}$ = 2.0, $J_{4,5a}$ = 5.9, $J_{4,5b}$ = 5.0), 2.057 (3H, H-1, s), 1.620

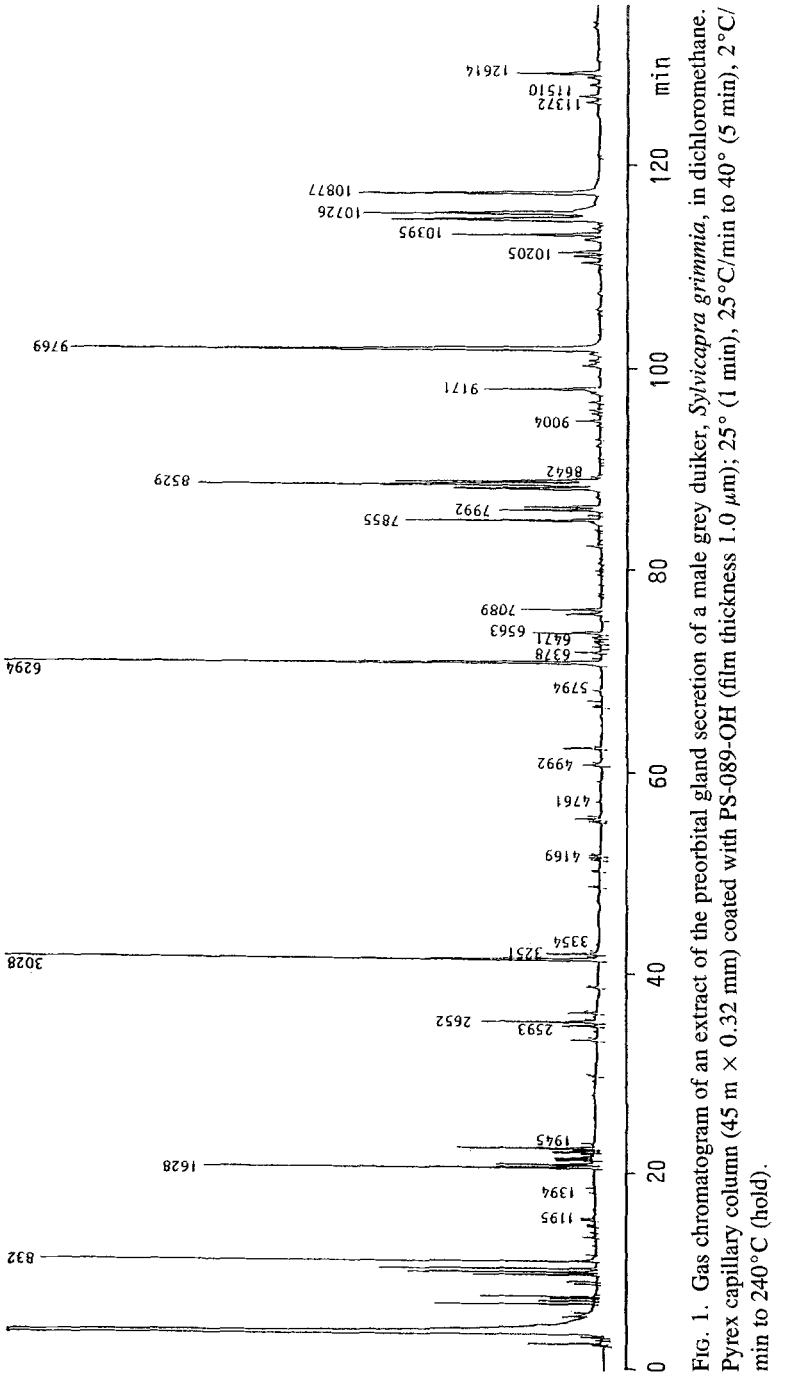


FIG. 1. Gas chromatogram of an extract of the preorbital gland secretion of a male grey duiker, *Sylvicapra grimmia*, in dichloromethane. Pyrex capillary column (45 m × 0.32 mm) coated with PS-089-OH (film thickness 1.0 μm); 25° (1 min), 25°C/min to 40° (5 min), 2°C/min to 240°C (hold).

(2H, H-5, m), 1.466 (2H, H-6, m), 1.275 (10H, H-7 to H-11, CH_2 envelope), 0.883 (3H, H-12, t, $J_{11,12} = 6.7$); [^{13}C]NMR ($CDCl_3$): δ 24.31 (q, C-1), 205.99 (s, C-2), 59.97 (d, C-3), 58.08 (d, C-4), 31.82 (t, C-5), 25.81 (t, C-6), 29.18 (t, C-7), 29.29 (t, C-8), 29.44 (t, C-9), 31.85 (t, C-10), 22.67 (t, C-11), 14.09 (q, C-12).

2-Isobutyl-4,5-dihydro-1,3-thiazole was synthesized (Burger et al., 1988) according to the procedure described by Wenker (1935) for 2-methyl-4,5-dihydro-1,3-thiazole. In the final step of this synthesis phosphorus pentasulfide (8.9 g, 20 mmol) and *N*-(2-hydroxyethyl) isovaleramide (14.5 g, 100 mmol) were mixed in a distilling flask fitted with an efficient cooler. The mixture was heated in an oil bath at 180°C, at which temperature the reaction took place and the required reaction product distilled from the flask at reduced pressure (110 torr). The crude product was dried on KOH and distilled to give the pure 2-isobutyl-4,5-dihydro-1,3-thiazole in a yield of 28%, bp 79°C/10 torr [lit. 70–73°C/10 torr (Meyers and Durandetta, 1975)]; [^{1}H]NMR ($CDCl_3$): δ 0.94 (6H, d, $J = 6.5$ Hz), 2.03 (1H, m, $J = 6.8$ Hz), 2.40 (2H, dt, $J = 6.8$ Hz; $J = 1.7$ Hz), 3.28 (2H, t, $J = 8.2$ Hz), 4.22 (2H, tt, $J = 8.4$ Hz; $J = 1.6$ Hz). [^{13}C]NMR ($CDCl_3$): δ 22.32 (2C, q), 27.52 (d), 33.65 (t), 43.16 (t), 64.14 (t), 171.29 (s).

2-Isobutyl-1,3-thiazole was prepared by the dehydrogenation of 2-isobutyl-2,5-dihydro-1,3-thiazole with chloranil according to Dubs and Pesaro (1974). The product of this reaction was distilled to give 2-isobutyl-1,3-thiazole in a yield of 59%, bp 60–62°C/14 torr [lit. 66°C/10 torr (Dubs and Pesaro, 1974)]; [^{1}H]NMR ($CDCl_3$): δ 0.98 (6H, d, $J = 6.6$ Hz), 2.12 (1H, m, $J = 6.8$ Hz), 2.88 (2H, d, $J = 7.2$ Hz), 7.16 (1H, d, $J = 3.3$ Hz), 7.66 (1H, d, $J = 3.3$ Hz); [^{13}C]NMR ($CDCl_3$): δ 22.04 (2C, q), 29.52 (d), 41.96 (t), 117.71 (d), 142.00 (d), 169.80 (s).

RESULTS AND DISCUSSION

The grey duiker is adaptable and widely distributed in sub-Saharan Africa except in deserts and dense forests. They are normally present in savanna and woodlands and also flourish on cultivated land (Kingdon, 1982) where they are a nuisance in orchards and plantations.

As in the case of grysbok, grey duiker males mark actively with their preorbital glands, while this behavior is rare in females (Dunbar and Dunbar, 1979). Unlike grysbok, grey duiker do not maintain dung heaps so that preorbital secretion appears to be the major medium of olfactory signaling. They live in individual home ranges with the ranges of males overlapping those of one or more females. Females' ranges may overlap to some extent but they drive intruders of the same sex out. Males occupy more discreet ranges, the boundaries of which they defend (Dunbar and Dunbar, 1979).

The preorbital gland structure of the grey duiker has been described by Richter (1973). It is more complex than in most other duikers (*Cephalophus* spp.) and consists of pigmented and unpigmented areas. The secretion consists of a thick black melanin-containing component and a thin, yellowish clear liquid. Preliminary bioassays indicate that grey duiker respond to fresh preorbital marks as well as to those up to about two weeks old by sniffing or marking over them. No response was elicited by marks exposed to the air for four weeks. Whether or not signals deter potential rivals remains to be determined.

Although young hand-reared grey duikers are quite docile, the males in particular tend to become dangerous when adult. The captured animals are, on the one hand, extremely aggressive, while, on the other, they are also easily stressed and regular handling may result in a high stress-related mortality. This complicated the present project, since it was impossible to obtain material for analytical purposes on a regular basis or to collect material in sufficiently large quantities for the preparative gas chromatographic isolation of specific components.

As noted above, the preorbital gland secretion of the grey duiker is heterogeneous. It consists of water, mucus, a thin yellowish oil, and large concentrations of heavy waxy material in varying proportions. In contrast to the preorbital secretion of the grysbok, which, when introduced into the injector of a gas chromatograph without any preliminary clean-up, left only a solid dry residue, the grey duiker secretion left an oily residue that contaminated the injector liner and was also particularly detrimental to capillary columns. Getting quantitatively reproducible gas chromatograms was further complicated by the heterogeneous nature of the secretion, since it was virtually impossible to control the concentration of organic volatiles in a sample and thus the loading of the column with these volatiles. The quantitative comparison of the secretion of individual animals was therefore carried out with extracts of the organic material. A number of analyses, in which direct introduction of the pure secretion into the injector was employed, were nevertheless carried out, using a series of injector temperatures from 150°C up to 240°C, in order to determine the thermal stability of the volatile organic compounds in the secretion. The quantitative results of these analyses did not show any variations that could be correlated with the injector temperature, and the compounds under investigation were therefore expected to survive the GC separation and transfer through the GC-MS interface. A typical gas chromatographic analysis of an extract of the preorbital gland secretion in dichloromethane is shown in Figure 1.

Initial tentative identification of a number of the volatile organic constituents of the secretion was based on their mass spectra and the results of a comparison of their spectra with those in the available spectra library. Further corroboration of the molecular mass of the compounds was obtained by chemical ionization mass spectrometry and final proof of the proposed structures was

provided by the coinjection of the extract with authentic synthetic material. In a few cases where some of the members of a homologous series were not available, careful use was made of the retention time increments obtained under temperature programming conditions for the neighboring positively identified members of such a series. Since the mass spectra of some of the constituents contained insufficient or ambiguous information, some of the even relatively prominent constituents remained unidentified. The stereochemistry of the chiral components was not investigated in the present study.

The mass spectrum of component 6294 (Figure 2) has a weak (1.0%) ion at *m/z* 198, which, according to the M + 1 ion at *m/z* 199 in its CI spectrum, is the molecular ion of this compound. By high-resolution mass measurements this ion was found to correspond to the molecular formula $C_{12}H_{22}O_2$, while the ion at *m/z* 85 has the elemental composition $C_4H_5O_2$. The presence of these ions in the spectrum can be interpreted in terms of component 6294 being a γ-substituted γ-lactone. In the spectra of such long-chain γ-lactones, however, the ion *m/z* 85 carries a much higher proportion of the total ion current, while the ion at *m/z* 43 is slightly weaker. The relatively high abundance of the *m/z* 43 ion in the mass spectrum of component 6294 can also be interpreted in terms of the presence of a methyl ketone function in the compound which, to satisfy the elemental composition of the *m/z* 85 ion, would require the presence of an

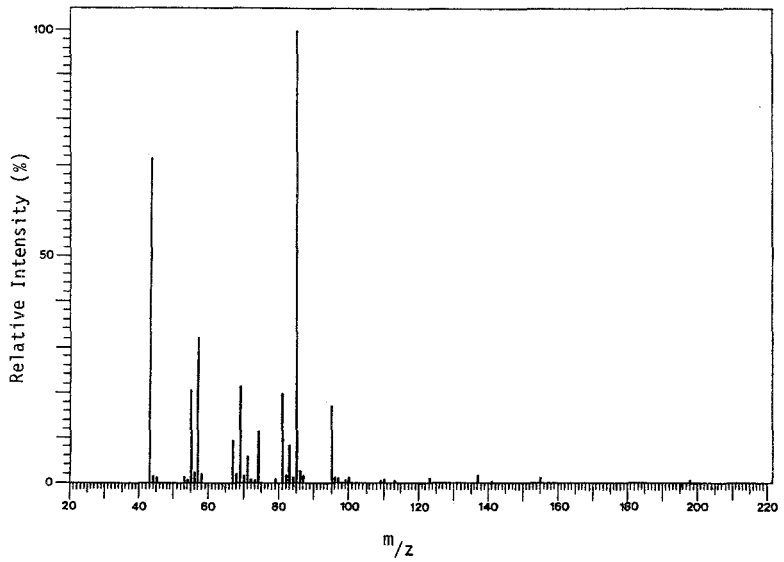
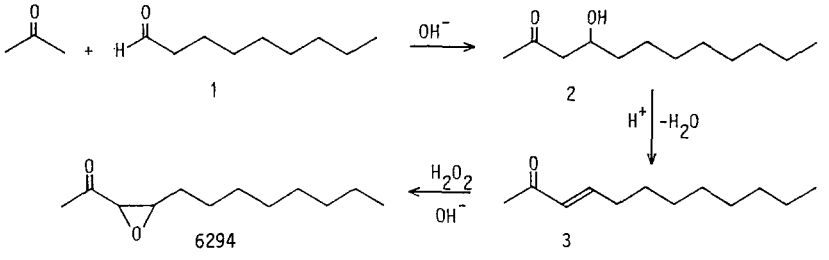


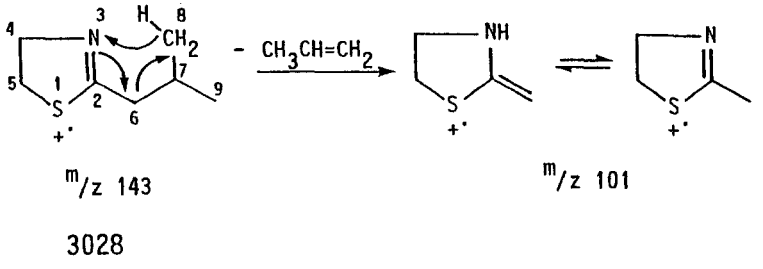
FIG. 2. Mass spectrum of component 6294 from the preorbital gland secretion of the grey duiker, *Sylvicapra grimmia*.

oxygen atom on one of the next two carbon atoms in the chain. 3,4-Epoxy-2-alkanones are known to have abundant ions at *m/z* 43 and 85 and weak molecular ions (Reusch and Djerassi, 1967), and component 6294 was therefore tentatively identified as 3,4-epoxy-2-dodecanone. In order to substantiate this assignment, this epoxy ketone was synthesized as shown in Scheme 1. The synthetic compound had a mass spectrum identical to that of the natural compound.



SCHEME 1.

The identification of 2-isobutyl-1,3-thiazole (2652) and 2-isobutyl-4,5-dihydro-1,3-thiazole (3028) in the preorbital secretions of the grey duiker and the red duiker (*Cephalophus natalensis*) has been discussed in detail in the preceding paper (Burger et al., 1988) in this series on mammalian pheromones. In summary, the assignment of these structures to the two compounds hinged on the presence of base peaks at *m/z* 99 and *m/z* 101 in the mass spectra of the compounds 2652 and 3028, respectively. The presence of the ion at *m/z* 101 in the spectrum of compound 3028, for example, was interpreted in terms of the rearrangement shown in Scheme 2, which can take place with either *n*-butyl or isobutyl in position 2 of a 4,5-dihydro-1,3-thiazole ring.



SCHEME 2.

The alkyl substituent in position 2 was finally unequivocally established to be an isobutyl group in both compounds by comparison of the mass spectra and gas chromatographic retention times of the natural compounds with those of authentic synthetic samples. Structural conclusions drawn from the mass spectra of these two thiazole derivatives were further substantiated by confirmation of their molecular ions by chemical ionization mass spectrometry and the determination of the elemental composition of the most abundant ions in the spectrum of the 4,5-dihydro-1,3-thiazole by high-resolution mass measurements.

The mass spectrum of 2-isobutyl-4,5-dihydro-1,3-thiazole, with a base peak at *m/z* 101, is distinctly different from that of 2-*sec*-butyl-4,5-dihydro-1,3-thiazole, which was found in urine of male mice (Liebich et al., 1977; Schwende et al., 1986) and has an abundant ion at *m/z* 115 instead of a base peak at *m/z* 101. The mechanism proposed for the formation of the base peak in the mass spectrum of the 2-isobutyl derivative can also be invoked to explain the presence of the ion at *m/z* 115 in the spectrum of the 2-*sec*-butyl derivative. This not only proves that these two compounds are distinctly different, but also lends support to the proposed fragmentation mechanism. As expected, these ions do not appear in the mass spectrum of the 2-isopropyl derivative, which is also present in male mouse urine.

Hexadecan-4-olide and octadecan-4-olide were prepared by the radical addition of acetic acid to 1-tetradecene and 1-hexadecene, respectively; oxidation of the resulting adduct radicals and cyclization of the carbocations was according to the method of Heiba et al. (1974) for the preparation of saturated γ-substituted γ-lactones.

The volatile compounds identified in the preorbital gland secretion are listed in Table 1. The majority of these compounds belong to one of four compound classes, viz. unbranched alkanes, 2-alkanones, alkanoic acids or alkan-4-olides, members of which have been identified in many mammalian secretions (Albone, 1984). Benzyl cyanide has been found in the chest gland secretion of the bush baby, *Galago crassicaudatus* (Crewe et al., 1979), and it has been suggested that in this animal it may have a function in short-term territorial marking. Based on the fact that this nitrile, the two γ-lactones, and the three sulfur-containing compounds, diethyl sulfoxide, 2-isobutyl-1,3-thiazole, and 2-isobutyl-4,5-dihydro-1,3-thiazole, as well as 3,4-epoxy-2-dodecanone are uncommon in mammalian exocrine secretions, they are expected to give the secretion a distintive character, although it cannot be ignored that some of the minor unidentified components of the secretion may be even more essential to its function. Since a reliable behavioral or electrophysiological bioassay is at this stage not yet available, it was impossible to use a response-guided strategy (Albone, 1984) for the identification of the compounds that influence the territorial behavior of the grey duiker. Although the alternative chemical image strategy was

TABLE 1. COMPOSITION OF VOLATILE ORGANIC FRACTION OF PREORBITAL GLAND SECRETION OF *Sylvicapra grimmia*

Peak[a]	Component	Quantity (μg)[b]	Identification/ criteria	Mass spectral data *m/z*(abundance, %)
832	3-Methyl-3-buten-1-ol	2.5	*c,d,f*	M^+: 86(41), 71(15), 68(86), 56(100), 41(90)
1195	2-Hexanone	0.8	*c,d,f*	M^+: 100(18), 85(11), 71(14), 58(52), 57(19), 44(11), 43(100)
1394	Octane	0.2	*c,f*	M^+: 114(13), 85(52), 84(32), 71(31), 57(33), 43(100)
1628	Diethyl sulfoxide	6.9	*c,d,f*	$M+2^+$: 108(11), 107(6), 105(100), 91(10), 76(5), 59(91), 45(16)
1945	Nonane	0.3	*c,f*	M^+: 128(11), 99(9), 85(37), 84(27), 71(25), 57(78), 43(100)
2593	Decane	0.2	*c,f*	M^+: 142(12), 113(5), 99(8), 85(34), 71(66), 57(91), 43(100)
2652	2-Isobutyl-1,3-thiazole	3.3	*c,d,f*	M^+: 141(3), 126(14), 99(100), 58(17), 43(6)
3028	2-Isobutyl-4,5-dihydro-1,3-thiazole	30.0	*c,d,e,f*	M^+: 143(8), 128(16), 101(100), 73(2), 60(65), 55(6)
3251	Benzyl cyanide	1.2	*c,d,f*	M^+: 117(100), 116(39), 90(33), 89(20), 51(7)
3354	Undecane	0.2	*c,f*	M^+: 156(9), 127(4), 113(6), 99(10), 85(36), 71(56), 57(100), 43(88)
4169	Dodecane	0.8	*c,f*	M^+: 170(10), 141(2), 127(4), 113(5), 99(10), 85(41), 71(64), 57(100), 43(85)
4761	2-Undecanone	0.3	*c,d,f*	M^+: 170(7), 155(6), 129(9), 115(11), 95(7), 85(12), 71(44), 58(100), 43(93)
4992	Tridecane	0.3	*c,f*	M^+: 184(9), 147(5), 129(9), 99(11), 85(43), 71(67), 57(100), 43(91)
5794	Tetradecane	0.6	*c,f*	M^+: 198(8), 175(3), 155(3), 141(5), 127(5), 113(7), 99(13), 85(44), 71(67), 57(100), 43(77)
6294	3,4-Epoxy-2-dodecanone	31.3	*c,d,e,f*	M^+: 198(1), 155(2), 137(3), 100(3), 95(19), 85(100), 81(20), 74(14), 69(22), 67(10), 57(32), 55(21), 43(73)
6378	2-Tridecanone	0.3	*c,d,f*	M^+: 198(7), 140(8), 127(3), 111(4), 96(9), 85(15), 71(43), 59(41), 58(100), 43(76)
6471	Tridecanal	1.4	*c,d,f*	169(17), 154(13), 137(9), 124(15), 109(21), 96(43), 82(76), 69(58), 57(100), 43(98)
6563	Pentadecane	2.2	*c,f*	M^+: 212(8), 169(19), 155(5), 141(5), 127(7), 113(8), 99(17), 85(55), 71(76), 57(100), 43(82)

17089	Dodecanoic acid	3.9	c,d,f	M^+: 200(17), 171(8), 157(29), 143(10), 129(51), 115(21), 101(17), 85(31), 73(100), 60(77), 55(49), 43(62)
17855	2-Pentadecanone	7.5	c,d,f	M^+: 226(8), 211(3), 183(1), 168(5), 138(2), 127(4), 111(4), 96(11), 85(15), 71(47), 58(100), 43(76)
17992	Heptadecane	0.3	c,f	M^+: 240(8), 169(3), 155(4), 141(5), 127(6), 113(9), 99(16), 85(58), 71(77), 57(100), 43(68)
18529	Tetradecanoic acid	1.9	c,d,f	M^+: 228(29), 199(5), 185(31), 171(11), 157(5), 143(14), 129(65), 115(18), 97(18), 85(24), 73(100), 60(71), 55(48), 43(62)
18642	Octadecane	0.3	c,f	M^+: 254(7), 228(6), 185(8), 169(3), 154(4), 141(7), 129(16), 113(12), 99(18), 85(56), 71(72), 57(100), 43(77)
19004	Pentadecanoic acid	0.8	c,d,f	M^+: 242(34), 213(6), 199(24), 185(18), 171(9), 157(10), 143(21), 129(58), 115(20), 97(32), 85(30), 73(100), 60(76), 55(74), 43(82)
19171	2-Heptadecanone	2.5	c,d,f	M^+: 254(9), 239(4), 225(1), 196(4), 166(1), 138(2), 127(5), 111(4), 96(15), 85(19), 71(50), 58(100), 43(78)
19769	Hexadecanoic acid	23.3	c,d,f	M^+: 256(50), 227(6), 213(26), 199(6), 185(15), 171(15), 157(18), 143(9), 129(58), 115(20), 97(24), 83(27), 73(100), 71(40), 60(69), 57(64), 43(76)
110205	Hexadecan-4-olide	0.8	c,d,f	M^+: 254(<1), 236(5), 218(3), 192(6), 166(3), 165(3), 152(7), 138(7), 125(22), 111(20), 97(37), 85(100), 83(32), 71(35), 69(39), 55(53), 43(70)
110395	2-Nonadecanone	1.1	c,d,f	M^+: 282(11), 267(4), 239(1), 222(4), 196(1), 166(1), 138(2), 127(6), 111(5), 96(18), 85(21), 71(57), 58(100), 43(84)
110726	(*Z*)-9-Octadecenoic acid	2.2	c,d,f	M^+: 282(5), 264(19), 222(6), 220(5), 180(5), 165(5), 151(7), 137(10), 123(15), 111(28), 97(59), 83(63), 69(82), 55(100), 43(54), 41(58)
10877	Octadecanoic acid	3.9	c,d,f	M^+: 284(69), 255(4), 241(27), 227(7), 213(4), 199(9), 185(26), 171(11), 157(5), 143(11), 129(65), 115(17), 97(31), 85(30), 83(35), 73(100), 57(76), 43(86)

TABLE 1. Continued

Peak[a]	Component	Quantity (μg)[b]	Identification/ criteria	Mass spectral data *m/z*(abundance, %)
11372	Octadecan-4-olide	0.6	c,d,f	264(12), 246(8), 220(10), 180(5), 165(5), 152(6), 138(6), 125(15), 111(24), 97(43), 85(100), 83(44), 71(29), 69(48), 55(59), 43(62)
11510	2-Heneicosanone	1.4	c,d	M^+: 310(14), 295(5), 267(1), 252(4), 222(1), 194(1), 166(1), 138(2), 127(6), 111(7), 96(23), 85(24), 71(59), 58(100), 43(87)
12614	2-Tricosanone	0.3	c,d	M^+: 338(14), 278(5), 236(2), 207(2), 138(2), 127(6), 111(8), 96(27), 85(25), 71(58), 59(100), 58(90), 43(85)

[a] Components numbered as in Figure 1.
[b] Quantities are given in μg/500 μg of secretion, which is an average quantity of secretion collected per animal from both glands.
[c] EI-MS.
[d] CI-MS.
[e] High-resolution MS.
[f] Coinjection with authentic synthetic compounds.

complicated by the limited number of animals available for this research, the variations in the composition of the preorbital gland secretions from individual animals were studied as a first step in this direction. The results obtained by quantitative analysis of the secretions from three males are given in Table 2. To obtain secretion from the male M1, the animal was netted on three occasions over a period of three weeks for the collection of material. The samples were pooled for analysis. The animal appeared to be sexually active during this period and was very aggressive. As a result of the collection of the material, the animal

TABLE 2. CONCENTRATION OF SELECTION OF CONSTITUENTS OF PREORBITAL GLAND SECRETIONS OF MALE AND FEMALE GREY DUIKERS NORMALIZED WITH RESPECT TO ARBITRARY CONCENTRATION OF 100% FOR HEXADECANOIC ACID PRESENT IN SECRETIONS

Peak no. in Figure 1	Component[a]	Relative concentration (%)					
		Males			Females[d]		
		M1[b]	M2[c]	M3[d]	$\bar{X}$	SD	Range[e]
1628	Diethyl sulfoxide	39.7	11.6	26.1	4.7	5.4	0.6–14.9
2652	2-Isobutyl-1,3-thiazole	29.0	106.0	28.1	3.1	2.7	0.9–7.4
3028	2-Isobutyl-4,5-dihydro-1,3-thiazole	81.4	150.8	52.2	5.2	2.9	1.9–8.5
3251	Benzyl cyanide	1.8	2.3	1.1	0.2	0.1	0.1–0.5
6294	3,4-Epoxy-2-dodecanone	85.0	24.7	23.6	3.3	1.3	1.5–4.9
6378	2-Tridecanone	2.2	3.8	1.7	4.0	2.0	1.0–6.8
6471	Tridecanal	4.0	13.1	5.1	4.0	1.1	2.2–5.1
7089	Dodecanoic acid	8.1	11.8	5.8	11.8	2.3	7.4–15.1
7855	2-Pentadecanone	18.5	6.5	15.0	18.4	3.5	12.8–22.9
8529	Tetradecanoic acid	53.5	22.6	13.9	28.0	5.0	18.0–31.4
9004	Pentadecanoic acid	2.0	3.9	4.1	1.0	0.4	0.6–1.9
9171	2-Heptadecanone	15.3	6.9	7.0	1.7	0.9	0.4–2.5
9769	Hexadecanoic acid	100.0	100.0	100.0	100.0	0	100
10205	Hexadecan-4-olide	1.3	1.0	1.2	1.0	0.2	0.7–1.3
10395	2-Nonadecanone	18.7	2.6	2.9	1.1	0.3	0.2–1.5
10726	(Z)-9-Octadecenoic acid	49.9	9.8	35.6	1.8	1.0	0.8–3.5
10877	Octadecanoic acid	45.9	<0.1	21.5	4.6	3.2	1.2–8.7
11510	2-Heneicosanone	0.9	<0.1	0.1	4.0	3.5	1.3–10.1
12614	2-Tricosanone	0.9	<0.1	<0.1	8.0	2.6	3.0–12.6

[a] The compounds are given in order of elution from the gas chromatographic column.
[b] Material scooped from the glands of a captive male.
[c] Material deposited by a captive male on clean aluminium foil.
[d] Material scooped from the glands of culled animals. Only M3 is directly comparable with the females.
[e] $N = 7$ females.

died of stress. Consequently material was obtained from the second male M2 by collecting freshly deposited territorial marks. A single sample of secretion was also obtained from a culled male M3. Since fresh secretion, collected directly from the animals, contains large and varying proportions of water in addition to the organic material, whereas deposited territorial marks appear to contain very little water, using an internal standard would have produced misleading results. Hexadecanoic acid, which is present in high concentrations in the secretions of both males and females, was therefore used as a quasiinternal standard, and the quantitative data were normalized with respect to this component of the secretion, taken as 100%. With the exception of a few components, such as 3,4-epoxy-2-dodecanone, tetradecanoic acid, and 2-nonadecanone, which are present in much lower concentrations in the secretion from the culled male M3 than in that from the male M1, the secretions from these two animals are similar. The standard, hexadecanoic acid, was apparently present in a slightly higher concentration in the secretion from the male M3, resulting in lower relative concentrations for almost all other constituents. The sample prepared by extracting the territorial marks deposited by the male M2 shows little resemblance to the other two samples. Diethyl sulfoxide, for instance, is present in a much lower concentration in this sample, whereas the two thiazole derivatives are the two major constituents of this secretion. Octadecanoic acid was present in a barely detectable concentration. Although some of the volatile material may have been lost by evaporation before the material could be collected, it is unlikely that this factor could account for these differences between this and the other two samples. Not too much significance can be attached to the results obtained from such a limited number of samples, but it is possible that in an attempt to collect as much material as possible, larger proportions of certain components are forced from the secretion-emitting pores than by the gentle rubbing motion with which the material is deposited by the animal. When the secretion is collected manually, the first material to emerge from the pores is a thin yellowish liquid, whereas a thicker black suspension is forced out of the pores when pressure is applied to the gland. Analysis of the territorial marks deposited by the animals is therefore expected to provide a more accurate volatile organic constituent profile of the secretion as it is employed for territorial marking by the grey duiker.

Although it was, unfortunately, impossible to carry out a full investigation of the seasonal variations in the quantitative composition of the preorbital secretion of male animals, analyses of the territorial marks deposited by the male M2 were carried out once a week over a period of eight weeks, the results of which are given in Table 3. This part of the study was undertaken two months after the analysis reported in Table 2. Once again hexadecanoic acid was used as quasiinternal standard. There does not seem to be a marked trend in the

TABLE 3. VARIATION IN CONCENTRATION OF SELECTED CONSTITUENTS OF PREORBITAL GLAND SECRETION DEPOSITED ON CLEAN ALUMINUM FOIL BY MALE GREY DUIKER M2[a]

Peak no. in Figure 1	Component[b]	Concentration (%)							
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1628	Diethyl sulfoxide	1.5	14.1	1.0	2.9	2.4	30.0	11.7	3.7
2652	2-Isobutyl-1,3-thiazole	10.7	14.8	10.0	57.7	50.5	26.0	22.2	23.4
3028	2-Isobutyl-4,5-dihydro-1,3-thiazole	26.0	37.7	23.5	88.5	94.1	65.4	43.0	44.5
3251	Benzyl cyanide	0.5	0.7	0.5	1.3	1.4	1.1	0.8	0.8
6294	3,4-Epoxy-2-dodecanone	2.2	0.9	4.1	3.5	4.1	8.0	6.9	8.6
6378	2-Tridecanone	1.2	3.3	1.1	8.2	12.4	6.3	5.3	5.9
6471	Tridecanal	3.4	0.7	2.7	1.0	2.0	0.5	0.6	0.4
7089	Dodecanoic acid	5.6	14.2	7.1	4.3	24.8	11.0	11.8	17.1
7855	2-Pentadecanone	5.6	8.0	5.8	2.5	4.2	8.1	2.6	3.5
8529	Tetradecanoic acid	22.1	10.5	17.1	2.9	4.8	15.8	21.8	21.9
9004	Pentadecanoic acid	9.1	2.9	4.3	3.0	4.2	3.7	1.0	0.8
9171	2-Heptadecanone	4.2	10.5	5.1	13.1	17.3	7.4	8.6	11.4
9769	Hexadecanoic acid	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
10205	Hexadecan-4-olide	1.0	1.2	1.1	1.3	1.4	0.9	1.1	0.8
10395	2-Nonadecanone	1.0	4.4	2.4	5.5	9.7	25.2	3.8	5.3
10726	(Z)-9-Octadecenoic acid	4.5	9.5	5.8	4.3	8.2	12.4	8.8	13.7
10877	Octadecanoic acid	1.2	2.1	1.5	16.8	3.3	0.5	2.4	2.9
11510	2-Heneicosanone	3.4	5.5	2.3	1.0	3.3	3.5	0.5	1.2
12614	2-Tricosanone	6.0	9.6	4.8	0.8	20.9	3.1	5.8	11.4

[a] The quantitative data are normalized with respect to an arbitrary concentration of 100% for the hexadecanoic acid present in the deposited material.
[b] Components are numbered as in Figure 1 and are given in order of elution from the gas chromatographic column.

relative concentrations of the components of the secretion except for diethyl sulfoxide, 2-nonadecanone, and 2-tricosanone, which reached a peak at about the sixth week with concentrations between 25 and 30 times as high as the lowest concentrations recorded in one of the previous weeks. One further point of interest is the remarkably parallel variation of the concentration of the two thiazole derivatives. The ratio of the relative concentrations of 2-isobutyl-1,3-thiazole and 2-isobutyl-4,5-dihydro-1,3-thiazole remained almost constant throughout the eight-week period.

Samples of material collected directly from seven culled females were likewise compared (Table 2). All the samples have very similar quantitative compositions. The differences that were found are considered to be too small to be of any significance in a semiochemical context. This result was anticipated since, as mentioned above, female grey duikers do not use their preorbital gland secretion for territorial marking.

A comparison of the data in Tables 2 and 3 reveals that 2-pentadecanone is present in higher concentrations in the female secretions than in the male secretions, whereas the situation is reversed in the case of 2-heptadecanone and 2-nonadecanone. The only consistent and significant difference between the secretions from the two sexes appears to be the much higher concentrations of the two thiazole derivatives and the epoxy ketone in the male preorbital secretions.

The dihydrothiazole derivatives, 2-isopropyl-4,5-dihydro-1,3-thiazole and the 2-*sec*-butyl-substituted analog, which are both closely related to 2-isobutyl-4,5-dihydro-1,3-thiazole, were identified as major constituents of the urine of male mice and were found to be either absent from the urine of females or present only in trace amounts (Liebich et al., 1977). The production of these compounds was found to be androgen dependent (Jemiolo et al., 1985; Schwende et al., 1986), to influence the frequency of estrus cycles in the females (Jemiolo et al., 1985), and to provoke male aggression (Novotny et al., 1985). It is unlikely that compounds such as the long-chain alkanes, ketones, and fatty acids, which are ubiquitous in mammalian exocrine secretions, play more than a supporting semiochemical role in the secretion and until evidence to the contrary has been obtained, no special significance can be attached to the observed variations in the concentrations of these constituents of the secretions. Further work on the territorial behavior of the grey duiker will therefore be concentrated on an investigation of the semiochemical as well as the physiological function of the two thiazole derivatives and the epoxy ketone that were identified in this secretion.

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METABOLISM AND EXCRETION OF THE FURANOCOUMARIN XANTHOTOXIN BY PARSNIP WEBWORM, *Depressaria pastinacella*

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Abstract—The parsnip webworm, *Depressaria pastinacella*, feeds on plants containing high concentrations of furanocoumarins, compounds toxic to many organisms. Parsnip webworm larvae were fed radiolabeled xanthotoxin to quantify the detoxification of this furanocoumarin. They metabolized approximately 95% of the ingested xanthotoxin, indicating that metabolic detoxification is important in their tolerance to this allelochemical. Excretion of xanthotoxin and its metabolites was not restricted to the frass but also occurred by means of the silk glands. The silk glands contained half as much of the tritiated compounds as the rest of the body. Because of the feeding habits of this insect, such an excretory pathway may have implications for interactions with predators and pathogens.

Key Words—*Depressaria pastinacella*, parsnip webworm, xanthotoxin, detoxification, furanocoumarins, silk, plant secondary compounds, plant-insect interactions, Oecophoridae, Lepidoptera.

INTRODUCTION

Furanocoumarins are plant secondary compounds most commonly found in the families Rutaceae and Umbelliferae (Murray et al., 1982). Upon exposure to long-wave ultraviolet light, many of these compounds are photoactivated and

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can bind covalently to nucleic acids and generate highly reactive oxygen species (Murray et al., 1982; Knox and Dodge, 1985). Because these processes can damage fundamental biological systems, furanocoumarins are toxic to a broad spectrum of organisms (reviewed in Murray et al., 1982).

A growing body of evidence indicates that furanocoumarins can be significant in herbivore–plant interactions. Not only are several furanocoumarins phototoxic to insect and mammalian herbivores (Berenbaum, 1978; Berenbaum and Neal, 1985; Ashkenazy et al., 1985), some also exhibit acute toxicity independent of photoactivation (Berenbaum, 1978; Berenbaum and Neal, 1985). Chronic toxicity as manifested by reduced fecundity can occur as well (Berenbaum and Feeny, 1981). In addition to postingestive effects, these compounds are frequently phagodeterrent (Yajima et al., 1977; Muckensturm et al., 1981). At a broader level of interaction, patterns of herbivore species distributions are correlated with furanocoumarin content in the Umbelliferae (Berenbaum, 1981). Moreover, the within-plant distribution of furanocoumarins is related to the relative value of the reproductive parts in one umbellifer species, suggesting that these toxins are allocated against herbivores in accordance with optimal defense theory (Nitao and Zangerl, 1987).

A well studied herbivore–plant interaction involving furanocoumarin chemistry involves the parsnip webworm, *Depressaria pastinacella* (Duponchel) (Lepidoptera: Oecophoridae). The larvae feed exclusively on plants in three genera of the Apiaceae, all of which contain furanocoumarins (Murray et al., 1982; Berenbaum, 1983). One of the webworm's major hosts in North America is the wild parsnip, *Pastinaca sativa* L. (Umbelliferae) (Hodges, 1974). In central Illinois, the wild parsnip generally produces six furanocoumarins, two of which, bergapten and sphondin, are associated with resistance of this plant against *D. pastinacella* (Berenbaum et al., 1984, 1986). Xanthotoxin, a furanocoumarin highly toxic to polyphagous insect herbivores (Berenbaum, 1978; Berenbaum and Neal, 1985), apparently is not a resistance factor against the parsnip webworm despite its abundance in the plant (Berenbaum et al., 1986). In fact, when bioassayed against the webworm in an artificial diet, xanthotoxin had no detectable effect on larval survivorship or development; in vitro studies suggested that *D. pastinacella* tolerance to xanthotoxin is due at least in part to metabolism by cytochrome P-450 monooxygenases (Nitao, 1989).

In this paper, I present the results of in vivo experiments ascertaining the extent to which the parsnip webworm detoxifies xanthotoxin and the physiological fate of ingested xanthotoxin and its metabolites. Previous studies on taxonomically unrelated insects that regularly feed on furanocoumarin-containing plants have found extensive in vitro and in vivo enzymatic degradation of xanthotoxin (Ivie et al., 1983; Bull et al., 1984, 1986; Ashwood-Smith et al., 1984). The mechanism by which the parsnip webworm tolerates this compound

should provide further information on how different herbivores cope with identical plant secondary chemicals.

METHODS AND MATERIALS

Metabolic Fate of Xanthotoxin. To quantify furanocoumarin metabolism by *D. pastinacella*, larvae were fed tritiated xanthotoxin (8-[*methoxyl*-^{3}H]psoralen, Amersham Corporation, Arlington Heights, Illinois; radiochemical purity of >99% verified by TLC). The tritiated furanocoumarin was diluted with unlabeled xanthotoxin (Sigma, St. Louis, Missouri) to a specific activity of 2.09×10^3 mCi/mmol in ethanol. Radiolabeled material in artificial diet (Nitao and Berenbaum, 1988) was presented to larvae in glass scintillation vials. One piece of artificial diet weighing approximately 0.2 g fresh mass was placed into each vial, and onto each piece was pipetted 10 μCi of [^{3}H]xanthotoxin in ethanol. The ethanol was evaporated with a stream of air, and a final (sixth) instar larva was placed into the vial. Larvae had been reared on artificial diet lacking xanthotoxin and were less than one day into the sixth instar when used; experimental individuals were offspring from field-collected insects (Champaign County, Illinois) (Nitao and Berenbaum, 1988). Rearing conditions were 25°C, 16:8 hr light–dark.

After three days, three larvae were dissected for quantification of the distribution of tritium in the body parts. The silk glands, the gut and its contents, the remaining carcass (including the fat body), and the frass from each larva, as well as the uneaten diet remaining in the vial, were frozen until analysis. The silk glands were briefly rinsed in 1.15% KCl in 0.05 M Tris HCl buffer (pH 7.8) prior to freezing to wash off hemolymph. Scintillation fluid was added to the used, empty vials and residual tritium quantified using standard liquid scintillation counting (LSC) procedures. The liquid scintillant consisted of dioxane, 300 g/liter naphthalene, and 15 g/liter PPO (2,5-diphenyloxazole). All readings were corrected for quench.

Xanthotoxin and its metabolites were extracted and quantified by using a procedure modified from Bull et al. (1984). Body parts and frass were homogenized in 1 ml of distilled water acidified to pH 2 with HCl. This homogenate was then extracted three times with 1-ml aliquots of ethyl acetate. The ethyl acetate fractions were combined, and aliquots were taken from both aqueous and ethyl acetate fractions for LSC. Preliminary work with unlabeled compound indicated that xanthotoxin partitions completely into the ethyl acetate phase as measured by high-pressure liquid chromatography. Hence, to determine the amount of unmetabolized xanthotoxin in the frass and body parts, only the ethyl acetate fractions were further analyzed. Ethyl acetate fractions were evaporated

to dryness, and the residues were redissolved in a small volume of ethyl acetate for spotting on silica gel thin-layer chromatography (TLC) plates (Eastman Chromagram sheet 13181). Extracts were cospotted with unlabeled xanthotoxin. TLC plates were developed in 75:25:1 ethyl acetate–methanol–acetic acid and visualized under long-wave UV to establish the R_f of xanthotoxin. TLC lanes for each extract were cut at 0.5-cm intervals and counted for tritium using LSC. The amount of radiolabel ingested was determined by subtracting the quantity of tritium in the uneaten diet and the residue remaining in the feeding vial from the initial amount given to the larva.

Excretion of Xanthotoxin and Metabolites in Silk. Parsnip webworms normally web together the flowers of their host plants and therefore produce large amounts of silk. Preliminary observations suggested that furanocoumarin and metabolites were excreted into the silk. Xanthotoxin and its major metabolites fluoresce under long-wave ultraviolet light (Bull et al., 1984); the presence of these compounds in webworm silk was suggested by the fluorescence of silk spun by furanocoumarin-fed larvae, whereas silk from larvae fed diet totally lacking in furanocoumarins did not fluoresce.

To verify that the presence of metabolites and furanocoumarin in the silk was not due simply to regurgitation or contamination from frass, the dispersion of these compounds in the silk was examined qualitatively. Silk was collected from the sides of a plastic cup in which three to five larvae had spun silk. These larvae were raised on 0.3% xanthotoxin (w/w) fresh mass diet from egg hatch through the early ultimate instar, starved for 8 hr to maximize clearance of the digestive tract, and then allowed to spin silk onto the sides of clean 28-ml cups. The silk was then collected and placed under long-wave ultraviolet light to visualize fluorescent metabolites and xanthotoxin. Dispersion of fluorescent material in globules or highly localized patches would indicate regurgitate or frass; uniform fluorescence throughout the silk fibers would suggest excretion of the compounds through the silk glands. Silk from larvae raised on control diet lacking xanthotoxin was also examined. The silks were placed under long-wave ultraviolet light and photographed through a No. 8 medium yellow correction filter.

To quantify the xanthotoxin and its metabolites in the silk, three larvae were fed [^{3}H]xanthotoxin for three days as described earlier. At the end of the three days, larvae were transferred to clean 28-ml plastic cups where they spun silk onto the cup wall. Prior to transfer, larvae were ligated with thread to close off the anus so as to avoid contamination of the silk with frass. After 8 hr, larvae were removed carefully so as to prevent defensive regurgitation, which would contaminate the silk, and the silk was collected. The silks were extracted sequentially for 1 hr each in 1 ml of ethyl acetate at room temperature, followed by 1 ml of 70% aqueous ethanol at 60°C. Silks were then solubilized by digestion in 100 μl of protease type XXV (Sigma, St. Louis, Missouri) (4 mg pro-

tease/ml 10 mM Tris buffer pH 7.6, 1 mM EDTA) at 37°C overnight. The radiolabeled material was quantified in all three fractions. The ethyl acetate extracts were evaporated to dryness and brought back up in a small volume of the same solvent to spot on TLC plates. The 70% ethanol extracts were also evaporated to dryness but were redissolved in absolute ethanol for application onto TLC plates. Plates were developed and analyzed as described earlier.

RESULTS

Metabolic Fate of Xanthotoxin

Recovery efficiency of the ingested tritium from larval body parts and the frass was approximately 90%. Nearly 95% of the ingested [^{3}H]xanthotoxin was metabolized; most of the unmetabolized [^{3}H]xanthotoxin was excreted with the frass (Table 1). Metabolites excreted in the frass accounted for approximately 80% of the recovered radiolabeled compounds out of the total tritium consumed. Xanthotoxin constituted less than 0.5% of the total ingested radiolabel present in the body exclusive of gut and gut contents; the silk glands by themselves contained half as much radiolabeled as the rest of the carcass. Most of the tritium in the silk glands was present in the form of metabolites.

Approximately 70% of the metabolites were recovered in the aqueous fraction, indicating that xanthotoxin was converted primarily into products of greater polarity. TLC analysis of the ethyl acetate fractions revealed that most metabolites partitioning into the organic phase had lower R_f values than that of xanthotoxin (R_f 0.70–0.75) (Figure 1). The lower R_f values on silica gel suggest that the ethyl acetate soluble metabolites were also generally more polar than

TABLE 1. DISTRIBUTION OF TRITIATED XANTHOTOXIN AND METABOLITES IN BODY PARTS AND FRASS OF *Depressaria pastinacella* SIXTH-INSTAR LARVAE[a]

	Ethyl acetate fraction			
	Unmetabolized [^{3}H]xanthotoxin	^{3}H metabolites	Aqueous fraction ^{3}H metabolites	Total
Silk glands	0.02 ± 0.008	1.76 ± 0.613	0.18 ± 0.117	1.96
Gut and contents	0.10 ± 0.058	5.46 ± 1.640	0.68 ± 0.118	6.24
Carcass	0.41 ± 0.220	2.81 ± 0.010	0.75 ± 0.175	3.87
Frass	6.41 ± 2.911	16.63 ± 4.976	62.83 ± 8.674	85.87
Total	6.94	26.66	64.44	

[a] Values are mean percent ± SE of ingested [^{3}H]xanthotoxin, $N = 3$.

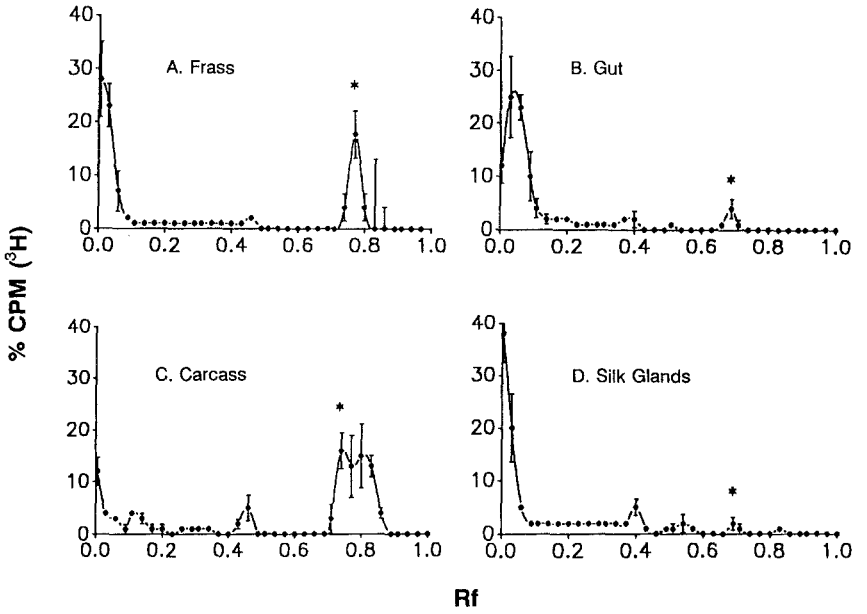

FIG. 1. Thin-layer chromatography of ^{3}H compounds in the ethyl acetate fractions of the frass and body parts from [^{3}H]xanthotoxin-fed *Depressaria pastinacella*. The x axis represents the R_f of the compounds; the y axis represents the percentage of ^{3}H detected as a percent of counts found in each ethyl acetate extract. *indicates R_f of xanthotoxin. Error bars are SE, $N = 3$.

xanthotoxin. The carcass, however, contained compound(s) having an R_f of 0.81 as well.

Excretion of Xanthotoxin and Metabolites in Silk. Silk collected from sixth-instar larvae raised on xanthotoxin diet fluoresced under long-wave ultraviolet light whereas silk from larvae reared on control diet did not (Figure 2). Moreover, the fluorescent compounds were dispersed uniformly throughout the silk and were not in localized patches or blotches, as would be expected if the compounds originated from regurgitant or frass.

The silk of larvae fed [^{3}H]xanthotoxin contained 0.10% ± 0.009 SE of the total tritium consumed and was mostly extracted in the hot 70% ethanol extract. Approximately 30% of this tritium was in the form of xanthotoxin (Table 2). No radiolabeled compounds were found in the protease digest, indicating that the ethyl acetate and 70% ethanol treatments extracted all of the detectable xanthotoxin and its radiolabeled metabolites; hence, there was no indication that tritiated metabolites were bound to the silk in an unextractable

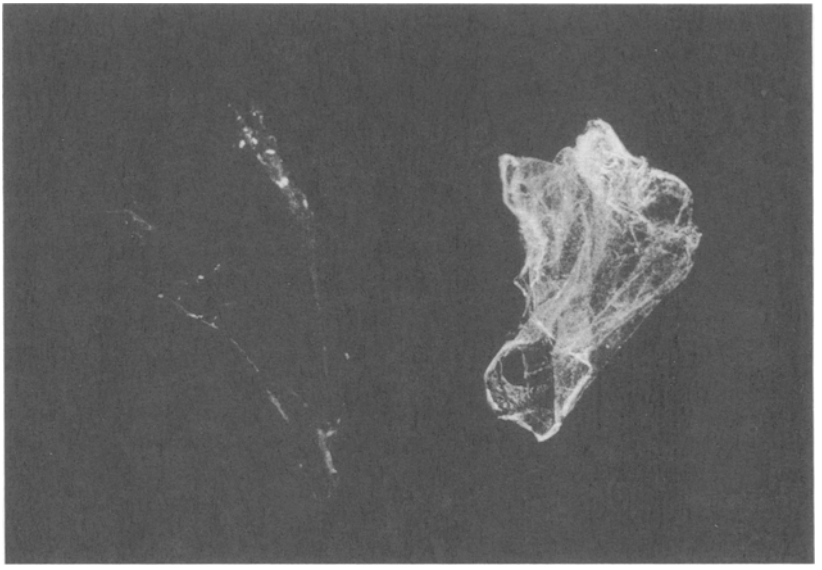

FIG. 2. Fluorescence under long-wave ultraviolet light of silk collected from *Depressaria pastinacella* fed control diet lacking xanthotoxin (left) and diet containing 0.3% w/w fresh mass xanthotoxin (right).

form. The mean mass of the collected silk was 0.21 mg ± 0.027 SE. Assuming that unlabeled and radiolabeled xanthotoxin are excreted into the silk in equal proportion, the estimated concentration of unmetabolized xanthotoxin in the silk was 1.16×10^{-4}%. As with the frass and body parts, most of the metabolites had lower R_f values than xanthotoxin (Figure 3).

TABLE 2. AMOUNT OF TRITIATED XANTHOTOXIN AND METABOLITES IN ETHYL ACETATE AND 70% ETHANOL EXTRACTS OF SILK COLLECTED FROM SIXTH-INSTAR *Depressaria pastinacella* LARVAE[a]

Extract	Unmetabolized [^{3}H]xanthotoxin	^{3}H metabolites
Ethyl acetate	0.007 ± 0.0023	0.005 ± 0.0003
70% aq. ethanol	0.017 ± 0.0026	0.077 ± 0.0167

[a]Values are percent ± SE of ingested [^{3}H]xanthotoxin, $N = 3$.

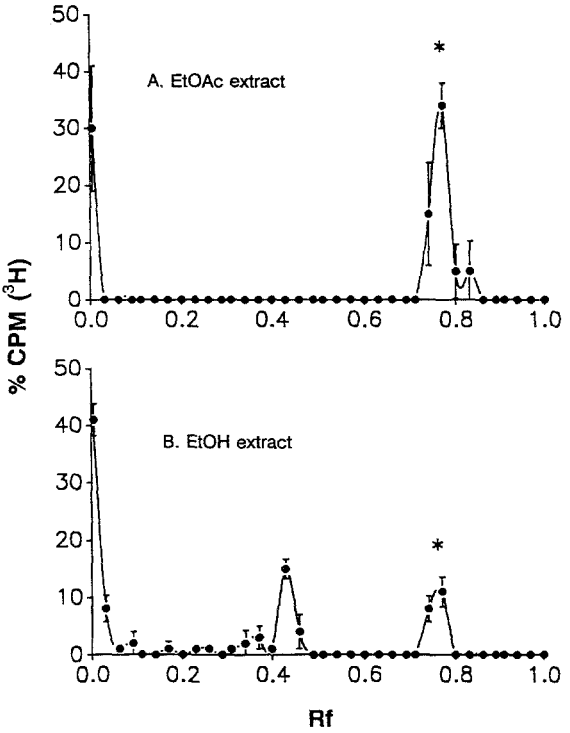

FIG. 3. Thin-layer chromatography of ^{3}H compounds in the ethyl acetate (EtOAc) and 70% aqueous ethanol (EtOH) extracts of silk collected from [^{3}H]xanthotoxin-fed *Depressaria pastinacella*. The *x* axis represents the R_f of the compounds; the *y* axis represents the percentage of ^{3}H detected as a percent of counts found in each extract. *indicates R_f of xanthotoxin. Error bars are SE, $N = 3$.

DISCUSSION

Insect herbivores avoid the deleterious effects of toxic plant secondary compounds through a variety of mechanisms. They may rapidly excrete the toxin unaltered, sequester the compound in specialized structures, metabolize the compound to an excretable form, or possess target sites physiologically insensitive to the toxin (Brattsten, 1986). In the parsnip webworm, biochemical metabolism appears to play a major role in conferring resistance to xanthotoxin. Approximately 95% of the ingested tritium recovered from larvae and frass was in the form of metabolites. These results are consistent with in vitro studies demonstrating that *D. pastinacella* microsomal enzymes possess high specific activity towards xanthotoxin (Nitao, 1989). The occurrence of unmetabolized

xanthotoxin in the body still presents the potential for toxicity; Lee and Berenbaum (in preparation) have found antioxidative enzymes in this insect and suggest that these enzymes protect against reactive oxygen species formed by furanocoumarin photoactivation.

Duffey (1980) discusses the possibility that the physiological fate of ingested plant secondary compounds is determined by the physical and chemical properties of the compound (e.g., polarity, solubility, binding constants). In other words, compounds may be predisposed to certain resistance mechanisms because of their inherent chemical properties. An expected outcome of this hypothesis is that taxonomically unrelated specialists will possess similar resistance mechanisms against the same compounds. Consistent with this prediction, sequestration is widespread in the various insect herbivores that feed on plants containing iridoid glycosides (Bowers and Puttick, 1986), cardenolides, and pyrrolizidine alkaloids (reviewed in Duffey, 1980). In contrast to these compounds, metabolic detoxification is common in the insects examined regularly feeding on furanocoumarin-containing plants. The black swallowtail, *Papilio polyxenes* (Lepidoptera: Papilionidae) (Ivie et al., 1983; Bull et al., 1984, 1986), and an agromyzid leaf miner, *Phytomyza spondylii* (Diptera) (Ashwood-Smith et al., 1984), as well as the parsnip webworm metabolize xanthotoxin. This toxin may therefore be more prone to oxidative metabolism and less amenable to sequestration.

In *D. pastinacella*, the metabolism of xanthotoxin is typical of xenobiotic detoxification in that it results in products more polar than the original compound. The presence in the carcass and silk of metabolites with slightly higher R_f than xanthotoxin on silica gel TLC is difficult to interpret since polarity and small differences in R_f are not perfectly correlated. A time–dose study would clarify the pharmacokinetics of xanthotoxin metabolism and help to ascertain whether these metabolites are intermediates in the detoxification process.

Xanthotoxin and its metabolites were found in the silk and silk glands; in fact, the silk glands by themselves contained as much of the ingested tritiated compounds as the rest of the body, which included the fat body and much of the hemolymph. Because the silk glands constitute only a minor fraction of the total body mass, the amount of xanthotoxin and metabolites was disproportionately high in these organs. As in many lepidopterous larvae, the glands were elongate, extending posteriorly along the digestive tract and terminating in the hindgut region. The extensive exposure to the hemolymph and orientation alongside the gut may predispose the silk-forming organs to absorb ingested allelochemicals and metabolites. Bergapten, another furanocoumarin, is also excreted in the silk (unpublished data), indicating that this process is not limited to xanthotoxin in *D. pastinacella*.

The larvae of hymenopterous parasitoids excrete nicotine and α-terthienyl in their cocoon silk when their lepidopteran hosts have been fed these plant

secondary compounds (Barbosa et al., 1986; McDougall et al., 1988). Other lepidopteran larvae are also known to excrete plant compounds into their silk (Brunet and Coles, 1974; Stermitz et al., 1988). This excretion pathway for allelochemicals therefore may be widespread in silk-producing insects that encounter xenobiotics. However, contamination by regurgitant, frass, or meconium can be a confounding factor in determining the occurrence of silk gland excretion (e.g., McDougall et al., 1988). In the study presented here, such contamination was unlikely. Visual examination of the silk under ultraviolet light revealed that the xanthotoxin and metabolites were dispersed uniformly throughout the silk strands and not in globules. Moreover, thin-layer chromatograms of the silk extracts in the radioisotope studies did not correspond with those of the gut and gut contents (Figures 1 and 3) as would be expected if the presence of radiolabel in the silk was due to regurgitant.

The incorporation of plant secondary compounds into silk may have functions other than as an elimination mechanism. Even the seemingly low xanthotoxin concentration of $1 \times 10^{-4}\%$ found in this study can have ecological implications. In nature, parsnip webworm larvae reside within silk-webbed flowers. Because the larvae are especially sensitive to ultraviolet light (personal observation), the incorporation of UV-absorbing compounds into the silk may serve to shield webworms from harmful solar radiation. Moreover, low concentrations of xanthotoxin inhibit fungal growth (Johnson et al., 1973; Camm, et al. 1976). The excretion of an antimicrobial agent in both frass and silk may produce a pathogen-free zone for the larva. Insect pathogens are known to spread and reside in the silk of some lepidopteran larvae (Jeffords et al., 1987), so that the presence of an antibiotic in the silk may prevent the establishment of deleterious microbes. Antimicrobial agents may be especially important in the high-humidity and frass-filled environment of the webs. Furthermore, the concentration of xanthotoxin in the floral parts of *P. sativa* can be over 300 times higher than that fed to larvae in the radioisotope experiments reported here. Thus, if the incorporation of xanthotoxin in the silk is dependent on dietary concentration, the levels in silk from a caterpillar feeding on a plant may be much higher. Finally, Stermitz et al. (1988) suggest that the presence of allelochemicals in silk may have antipredator functions. At higher concentrations, furanocoumarins may make the silk a chemical barrier as well as a physical one, deterring predators and parasites that probe or chew through the silk to reach larval or pupal hosts.

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Andrena wilkella MALE BEES DISCRIMINATE BETWEEN ENANTIOMERS OF CEPHALIC SECRETION COMPONENTS

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Abstract—Diastereomers of the spiroacetal, 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, represent main components of the cephalic secretion from males of the solitary bee, *Andrena wilkella*. The major compound proved to be of high enantiomeric purity, showing (2*S*,6*R*,8*S*) configuration. Only the naturally occurring enantiomer attracted patrolling males in the field; its antipode was behaviorally inactive and in a racemic mixture did not inhibit response. The (*E*,*Z*) diastereomers were also found to be almost inactive. EAG studies gave the same result as the behavioral tests. The biological function of the spiroacetal is discussed in view of the evolution of the mating behavior in *A. wilkella*.

Key Words—Enantiomer discrimination, male patrolling, odor marking, Hymenoptera, Apoidea, *Andrena wilkella*, bee, EAG, spiroacetal, absolute configuration, 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane.

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INTRODUCTION

Chemical signaling plays an important role in the mating strategies of insects. In many species, the enantiomeric composition of chiral compounds released in volatile secretions is essential for species-specific signaling. Striking examples are reported from bark beetles. The ambrosia beetle *Gnathotrichus sulcatus* (Le Conte) uses a 65:35 mixture of (*S*)- and (*R*)-6-methyl-5-hepten-2-ol (sulcatol) and needs both enantiomers for full response (Borden et al., 1976), while the closely related *G. retusus* (Le Conte) produces (*S*)-sulcatol in at least 99% optical purity and does not respond to the racemate (Borden et al., 1980). In the case of *Ips pini* (Say), interpopulational differences in pheromone biology are based on different enantiomeric mixtures of the principal aggregation pheromone, ipsdienol (Lanier et al., 1980).

Males of the palaearctic bee, *Andrena wilkella*, deposit odor marks along their patrol routes using a volatile cephalic secretion. This is a complex mixture of spiroacetals, alcohols, hydrocarbons, and terpenes (Bergström et al., 1982), among which (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane [(*E*,*E*)-DSU] (**1** in Figure 1, which shows structures of oxygen-containing heterocycles mentioned in this paper), accompanied by minor amounts of its *E*,*Z*-isomer, is one of the main components. 2-Methyl-5-pentyl-3,4-dihydro-2H-pyran (2HPYR) (**2** in Figure 1) (Francke, 1984), biogenetically probably closely related to the spiroacetals, also occurs in major amounts.

The objectives of our study were to determine the absolute configuration of DSU in *A. wilkella* and to test whether males discriminate between the enantiomers of (*E*,*E*)- and (*E*,*Z*)-DSU. We report results from long-term field experiments on the behavioral responses elicited in patrolling bees and from electrophysiological (EAG) studies of the reactions of primary odor receptors to these oxygen-containing heterocycles.

METHODS AND MATERIALS

Investigations were carried out in May–June of 1983–1985 on Öland, an island close to the southeastern coast of the Swedish mainland. A small grazed meadow surrounded by deciduous woodland served as the arena for the behavioral studies. The electrophysiological analyses were performed at the Ecological Research Station.

Pure enantiomers and racemates of (*E*,*E*)- and (*E*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, as well as racemic 2-methyl-6-pentyl-3,4-dihydro-2H-pyran served as test compounds. Pure hexane was used as a control. The compounds were prepared according to previously described methods (Francke et al., 1980b; Mori and Tanida, 1981; Isaksson et al., 1984). All samples were

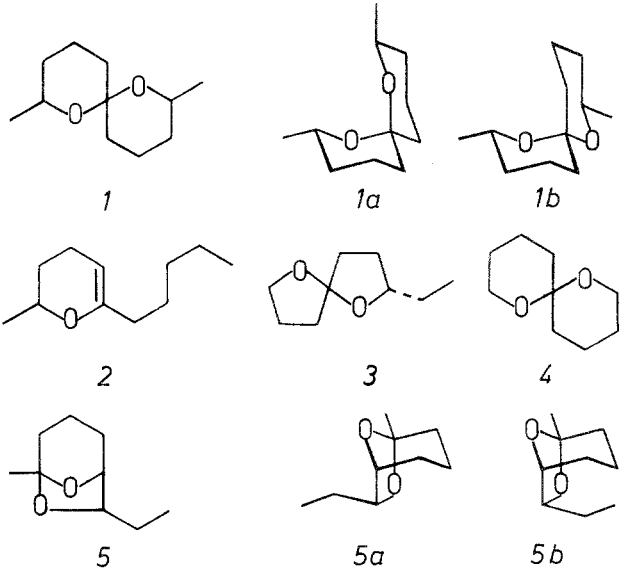

FIG. 1. Structural formulas of oxygen-containing heterocycles mentioned: **1** = 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (DSU); **1a** = (−)-(2*S*,6*R*,8*S*)-DSU [(−)-(*E*,*E*)-DSU]; **1b** = (−)-(2*R*,6*R*,8*S*)-DSU [(−)-(*E*,*Z*)-DSU]; **2** = 2-methyl-6-pentyl-3,4-dihydro-2H-pyran [2HPYR]; **3** = (2*S*,5*RS*)-ethyl-1,6-dioxaspiro[4.4]nonane (2*S*-chalcogran); **4** = 1,7-dioxaspiro[5.5]undecane (olean); **5** = 7-methyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane (brevicomin); **5a** = (+)-*exo*-brevicomin; **5b** = (+)-*endo*-brevicomin.

stored at −20°C prior to use. While the dihydropyran proved to be a little sensitive, the spiroacetals were stable under neutral conditions at ambient temperatures and showed no tendency to racemize. Despite the fact that complexation gas chromatography (Weber and Schurig, 1984) separates mostly enantiomers of bicyclic acetals, attempts to resolve (*E*,*E*)-DSU failed. Resolution was improved through the use of a modified cyclodextrin as a stationary phase (König et al., 1988a,b). More specifically in our case, successful separation was obtained by using perhexyl-α-cyclodextrin; conditions are given in the legend to Figure 2.

For the behavioral experiments, synthetic compounds (as 1% v/v solutions in hexane) were presented in test series to patrolling *A. wilkella* males. Each substance was included in either 16 or 23 test series replicates, depending on whether it was tested over two or three seasons, respectively. During a single test series, all test substances were presented successively and in random order for 5 min each. A velvet strip (6 × 10 mm) attached to an insect pin was

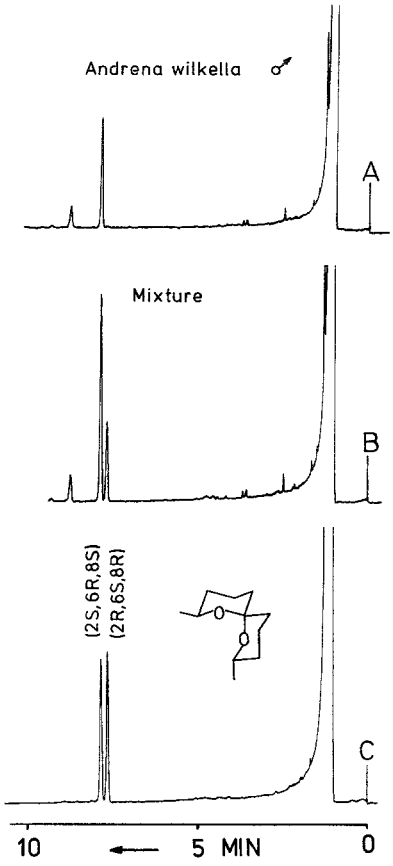

FIG. 2. Determination of the absolute configuration of DSU from *A. wilkella* by gas chromatography: per-*n*-hexyl-α-cyclodextrin; 35-m glass capillary; 0.25 mm ID; 80°C isothermal; 0.8 bar H2. (A) Natural extract; (B) natural extract spiked with racemic DSU; (C) resolution of racemic DSU.

impregnated (by soaking) with 0.1 ml of a test substance immediately before the experiment and was placed on a twig frequently patrolled by males. The behavioral responses of male bees were registered during the 5-min period, using the classification of Kullenberg (1973) and Tengö (1979): unaffected passage, quick approach, or pendulating/hovering inspection. The latter class represents the highest mood of excitement and thus reflects the highest biological activity of the test substance.

All experiments during each year were performed by one investigator. Two test series were carried out in parallel at sites 20 m apart by alternating test

periods between the sites. To minimize bias, the identity of the tested odor was never revealed to the investigator (blind test).

The number of males patrolling the test area differed between years, ranging approximately from 40 to 100. Individual males passed within a few meters of the test twig up to three times each 5-min period. Only test series with a minimum of five males passing within 10 cm of the odor source each 5 min were included in statistical analyses.

The attraction capacity of each substance was defined as the sum of the observations of hovering inspections (weighted with a factor 2) and quick approaches (unweighted), divided by the total number of behavioral events observed per substance. The resulting figure was standardized by assigning the substance with the highest attraction a value of 100 and the substance with the lowest attraction (i.e., the control) a value of 0. The behavioral patterns from the three years were tested for homogeneity using a χ^2 contingency test.

The response of four *A. wilkella* males to each of the test substances was electrophysiologically analyzed using electroantennogram techniques. The males were collected at midday and kept at 4–6°C for 1–3 hr until tested. The test substances were dissolved in distilled hexane (1 : 1000, v/v) and stored at −25°C between experiments. The antennograms were obtained by standard methods (Borg-Karlson et al., 1988). For sample preparation, 10 μl of each solution (equilibrated at ambient temperature) were applied to a piece of filter paper, which (after evaporation of hexane) was put into a glass vial placed inside a disposable plastic syringe; the odor-impregnated filter papers were replaced every hour. The EAG tests were performed at a temperature of 22.9–27.7°C and a relative humidity of 34–40%; hexane was used as the control, octanol as the standard.

Calculations of electrophsiological responses followed the method of van der Pers (1981), which corrects for the decreasing sensitivity of the antenna and for differences in sensitivity between animals, electrodes, and electrode positions.

The correlation between the behavioral and electrophysiological responses was evaluated using the Spearman rank method.

RESULTS

Gas chromatographic analyses revealed (*E,E*)-DSU to be secreted by *A. wilkella* males only as the (−)-enantiomer (Figure 2). The average amount per male ranged between 5 and 30 μg.

The behavioral data were similar over all three years (χ^2 contingency test, $P > 0.05$) and were consequently pooled for subsequent analyses (Figure 3). Each test substance differed significantly from the control in terms of the three

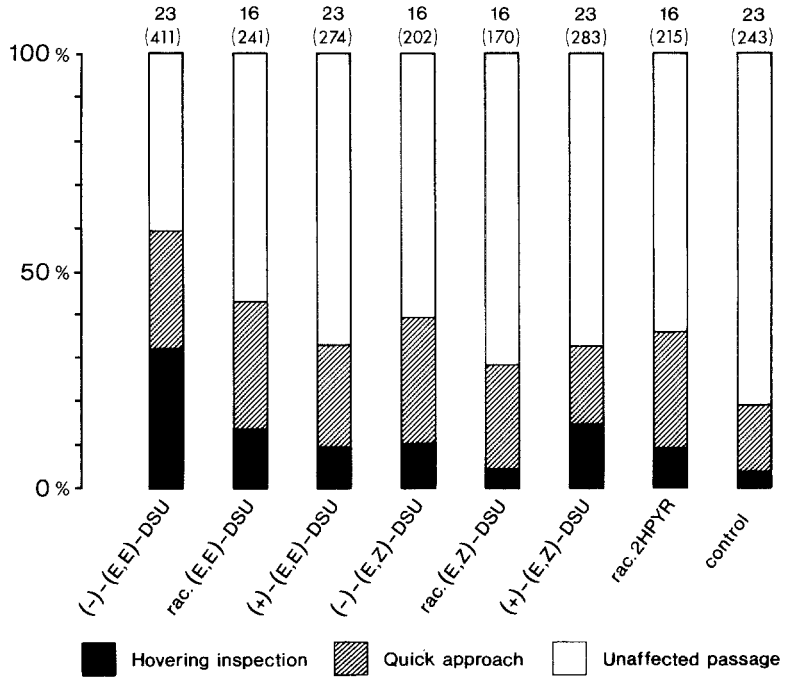

FIG. 3. Percentage of different behavioral responses (hovering inspection, quick approach, and unaffected passage) evoked by each odor substance in *A. wilkella* in field experiments. Number of test series replicates and total number of behavioral responses (in parentheses) are shown at the top of each bar.

behavioral response classes elicited in *A. wilkella* males (χ^2 test, $P < 0.05$, Table 1, Figure 3), and all evoked differing levels of excitement. When the substances were arranged in order of decreasing attraction capacity (Table 1), (−)-(*E*,*E*)- and racemic (*E*,*E*)-DSU took prominent positions. The responses evoked by each of these two compounds differed significantly from those of the other substances (χ^2 test, $P < 0.001$). The racemate elicited a slightly, but not significantly, weaker response in comparison with the (−)-enantiomer. The bees responded very weakly to (+)-(*E*,*E*)- and (+)-(*E*,*Z*)-DSU.

The behavioral response shown to racemic 2HPYR was significantly stronger than to racemic (*E*,*Z*)-DSU (χ^2 test, $P < 0.01$), but weaker than to racemic (*E*,*E*)-DSU (χ^2 test, $P < 0.05$, Figure 3).

EAG responses were highest to (−)-(*E*,*E*)- and racemic (*E*,*E*)-DSU (Table 1). Responses to the other substances were markedly lower; in only one case [racemic (*E*,*Z*)-DSU] were they significantly different from the control. The

TABLE 1. ATTRACTION CAPACITY (IN DECREASING ORDER) OF ODOR SUBSTANCES TESTED IN THE FIELD AND RELATIVE EAG RESPONSES

Odor substance[a]	Attraction capacity (%)[b]	Relative EAG response (concentration 1:1000)[c]		
		Mean[d]	S.D.	P^e
(−)-(*E*,*E*)-DSU	100	1.13	0.38	0.01
rac-(*E*,*E*)-DSU	84.3	1.07	0.43	0.025
(−)-(*E*,*Z*)-DSU	68.6	0.24	0.18	N.S.
rac-2HPYR	58.6	0.04	0.07	N.S.
(+)-(*E*,*Z*)-DSU	31.4	0.01	0.01	N.S.
rac-(*E*,*Z*)-DSU	30.0	0.19	0.07	0.025
(+)-(*E*,*E*)-DSU	22.9	0.10	0.16	N.S.
Control	0	0	0	—

[a] See Introduction.
[b] See Methods and Materials.
[c] Following van der Pers (1981).
[d] Values from four males.
[e] Probability of error that the relative EAG response differs from the control (*t* test) (N.S. = not significant).

relative EAG response to each substance was positively correlated with the behavioral attraction capacity, showing the highest values for (−)-(*E*,*E*)- and racemic (*E*,*E*)-DSU (Spearman rank correlation, $r_s = 0.81$, $N = 8$, $P = 0.015$).

The short seasons only permitted tests at one concentration. However, single test series replicates using 0.1 and 0.01% v/v solutions in hexane showed the same trend as 1% solutions.

DISCUSSION

Bicyclic Acetals in Systems of Chemical Communication. Bicyclic acetals are widespread among insect volatiles and have been reported as pheromone components of beetles, flies, bees, and wasps. As in many other cases, the enantiomeric composition of these compounds plays a major role in the generation of an efficient olfactory signal. Some of these compounds originate from the acetate pool, since they show an unbranched carbon skeleton.

The *exo*- and *endo*-isomers of brevicomin, 7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane (**5** in Figure 1), were first identified from frass of female western pine beetles, *Dendroctonus brevicomis* (Silverstein et al., 1968). Natural *exo*-brevicomin, the female-released aggregation pheromone of *D. brevi-*

comis, was found to have (1*R*,5*S*,7*R*) configuration (Stewart et al., 1977), and the beetles were shown to be able to discriminate between the enantiomers (Wood et al., 1976). Complexation gas chromatography (Weber and Schurig, 1984) of abdominal volatiles of male mountain pine beetles, *D. ponderosae*, revealed the presence of (1*R*,5*S*,7*R*)-(+)-*exo*-brevicomin (**5a** in Figure 1) of high enantiomeric purity; enantiomeric proportions of the additionally produced (1*R*,5*S*,7*S*)-(+)-*endo*-isomer (**5b** in Figure 1) ranged between 65 and 75% enantiomeric excess, with some variation evident between populations (Schurig et al., 1983). In *Dendroctonus frontalis*, (1*R*,5*S*,7*S*)-*endo*-brevicomin is the natural attractant in the male-produced aggregation pheromone (Redlich et al., 1987); its antipode inhibits response (Vité et al., 1985).

A spirocyclic acetal, a component of the male pheromone in the bark beetle, *Pityogenes chalcographus*, is 2-ethyl-1,6-dioxaspiro[4.4]nonane (chalcogran, **3** in Figure 1), which occurs as a mixture of diastereomers (Francke et al., 1977). The natural product shows (2*S*) configuration (Schurig and Weber, 1984), and the racemate is attractive in the field. In the olive fly, *Dacus oleae*, the active constituent of the sex pheromone is 1,7-dioxaspiro[5.5]undecane (**4** in Figure 1) (Baker et al., 1980). The females produce a racemate; however, males and females react differently to the enantiomers (Haniotakis et al., 1986).

Enantiomeric Discrimination in Hymenoptera. Although many chiral compounds have been identified from Hymenoptera (Blum, 1981), the absolute configurations have been determined in only some. Still less is known about enantiomeric discrimination at the receptor site. In ants, the classical case is 4-methyl-3-heptanone, an alarm pheromone of *Atta* spp.: the ants produce the (*S*) enantiomer, and only this isomer is active (Riley et al., 1974). Certain *Myrmica* spp. produce (3*R*)-octanol of high enantiomeric purity and react only to the natural product (Attygalle et al., 1983; Attygalle and Morgan, 1984).

Few cases of enantiomer discrimination are known from bees. Recently, it has been reported that enantiomeric composition of (2*E*)-9-hydroxy-2-decenoic acid is important for the retinue response to queen honeybees (Slessor et al., 1988; Winston et al., 1982). The stingless bee, *Scaptotrigona postica*, produces methylcarbinols of high optical purity (Engels et al., 1987) and the bees react differently to the enantiomers (W. Engels, personal communication).

2,8-Dimethyl-1,6-dioxaspiro[5.5]undecane (**1** in Figure 1) seems to be particularly widespread as an insect volatile (Francke et al., 1980b; Tengö et al., 1982; Davies and Madden, 1985; Dettner and Schwinger, 1986; Kitching et al., 1986); nevertheless its biological function is still largely unknown. The present study clearly shows both a central role of the (2*S*,6*R*,8*S*) isomer [(−)-(*E*,*E*)-DSU] (**1a** in Figure 1) in the communication systems of the bee *A. wilkella* and the males' ability to discriminate between the enantiomers (which also smell different to the human nose). To our knowledge, this is the first time that

enantiomeric discrimination has been demonstrated in solitary bees. In the natural secretions, (*E,E*)-DSU is accompanied by its *E,Z* isomer in a proportion of approximately 3 : 1 (Bergström et al., 1982). Although the absolute configuration of the latter remains to be determined, the bioassay clearly showed that the attractivity of this minor compound was inferior and that here, too, there were distinguishable differences between its enantiomers.

Since the attractivity of single compounds to male bees can be enhanced in some *Andrena* species by the addition of compounds identified from the cephalic secretion (Tengö, 1979), we have also tested a mixture of racemic spiroacetals, terpenes, and open-chain acetogenins, which resemble the natural blend found in *A. wilkella* (Bergström et al., 1982). Comparative experiments with this mixture and (−)-(*E,E*)-DSU revealed no significant differences in attraction capacity (Tengö, unpublished data) and thus no synergism in male attraction.

Biological Function. Communication between mating partners in bees and wasps has evolved in parallel with the temporal and spatial distribution patterns of the sexes. In the cases where sexually receptive female bees form aggregates, males normally wait at the aggregation areas and often compete for the most optimal territories (Alcock et al., 1978; Eickwort and Ginsberg, 1980; Thornhill and Alcock, 1983). By contrast, when females are randomly distributed within the habitat, males may exhibit different searching strategies (Alcock et al., 1978). For example, males may aggregate at prominent landmarks—such "hilltopping" behavior is reported from several species by Alcock (e.g., 1987)—or may display a spatially more extended patrolling, as in many *Andrena* species (Tengö, 1979).

Andrena wilkella bees nest in a random pattern within areas where both preferred flowers and prominent landmarks are also randomly dispersed. Thus, the probability of a male finding a place with receptive females is low. *Andrena wilkella* males patrol distinctly separated bushes, shrubbery, or curtains of deciduous trees, such that their flight is confined to a specific part of available vegetation (Tengö, unpublished). Flight routes often overlap extensively, with males frequently passing a place simultaneously, in small swarms. The same mating-flight areas are patrolled year after year, as are the landmarks used by hilltopping species (Alcock, 1984).

Andrena males mark spots along their mating-flight routes with secretions from the mandibular glands (Tengö, 1979). Such odor marks, as well as behavior-releasing synthetic compounds, have been found to attract patrolling *Andrena* males in field tests (Tengö, 1979). It seems plausible that *A. wilkella* males enhance the chances of aquiring mates by announcing their presence with odor marks, despite the fact that both females and copulation initiations are rarely seen at the patrolled areas. Likewise, when several males mark the same area,

hence increasing the total odor emittance, and when receptive females are randomly dispersed within the habitat, this chemical advertising may increase the mating chances of each male.

A male repeatedly visits the spots he marks, an investment for successful mating. When he deposits his own odor marks near those of other males, this odor should optimally be species-specific, especially in cases when males of more than one bee species occur in the same habitat. Males of up to five *Andrena* species, all specific in the composition of their cephalic secretions, have been observed patrolling the same shrubbery at a single time (Tengö, 1979). Comparative analyses of *A. wilkella* and two other species of the subgenus *Taeniandrena* showed distinct differences in odor bouquets, although all contain DSU as a major component (Bergström et al., 1982).

The transmission of a unique odor signal at the intraspecific level can arise from either specific blends or the presence of unique substances, including enantiomeric compositions of chiral compounds. The cephalic secretions of *A. wilkella* fit both possibilities. The high optical purity of natural (−)-(*E*,*E*)-DSU, its attractivity to males, as well as the ability of males to discriminate between its enantiomers, emphasize the importance of this unique compound in male patrolling behavior.

In this context, it is noteworthy that males of an ichneumonid wasp, *Meringopus titillator*, sometimes found patrolling the same bushes as *A. wilkella* males, also secrete (*E*,*E*)-DSU (of unknown absolute configuration) from their cephalic glands (Tengö, Francke and Hinz, unpublished data). DSU and 2HPYR have been found in other ichneumonids (Davies and Madden, 1985).

The complexity of the cephalic secretion in *A. wilkella* (ca. 50 components; Bergström et al., 1982) points to multifunctionality; intraspecifically, it may act as an individual odor, containing information used in kin recognition (Greenberg, 1979; Smith, 1983). Indeed, careful gas chromatographic analyses of cephalic secretions shows variation between individuals (Francke and Tengö, unpublished data).

In conclusion, males of *A. wilkella* discriminate both behaviorally and electrophysiologically between the enantiomers of the major component of their cephalic secretion, (*E*,*E*)-DSU, thereby allowing acuity in the inter- and intraspecific communication to operate in their mating behavior.

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IMPACT OF ATMOSPHERIC POLLUTION ON LINEAR FURANOCOUMARIN CONTENT IN CELERY

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Abstract—In a series of laboratory studies, a single 4-hr acidic fog at pH levels associated with commercial celery (*Apium graveolens* L.) production near major population centers in California was found to stimulate development of psoralen, bergapten, xanthotoxin, and isopimpinellin within 24 hr and for at least 120 hr after exposure. At 120 hr posttreatment, the concentrations of phototoxin furanocoumarins (psoralen + bergapten + xanthotoxin) increased 540% in the leaves (to 135 μg/g fresh weight) and 440% in the petioles (to 55.56 μg/g fresh weight) of celery exposed to a pH 2.0 fog as compared to plants exposed to control fogs (pH 6.3–6.5). Concentrations of these compounds in test plants were 7.5 times higher than the amount known to produce contact dermatitis. The nonphototoxic isopimpinellin increased more than threefold in the leaves (to 39.23 μg/g fresh weight, 120 hr) and petioles (to 25.88 μg/g fresh weight) as compared to control plants. In contrast, a single ozone fumigation at 0.20 ppm for 2 hr generally reduced concentrations of phototoxin furanocoumarin in leaves of celery within 24 hr (ozone-treated plants = 37.9, controls = 69.5 μg/g fresh weight), but levels of these chemicals in leaves of ozone-fumigated plants increased rapidly and concentrations were not significantly different at 120 hr. Isopimpinellin concentrations in foliage followed a similar trend (at 24 hr, control = 25.11, ozone treated = 10.96 μg/g fresh weight, no difference at 120 h). In petioles, none of the linear furanocoumarin levels differed significantly at 120 hr posttreatment.

Key Words—Celery, *Apium graveolens*, atmospheric pollution, ozone, acidic fog, furanocoumarins, bergapten, xanthotoxin, psoralen, isopimpinellin.

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INTRODUCTION

Phototoxin dermatitis caused by contact with celery infected with a *Sclerotinia* fungi has been reported since the early 1900s (Austad and Kavli, 1983; Ashwood-Smith et al., 1985). Since the mid-1970s, the causal agents of the dermatitis have been definitively identified as linear furanocoumarins. When exposed to long-wave UV light, the phototoxic linear furanocoumarins (psoralen, xanthotoxin, and bergapten) rapidly alkylate DNA (Scott et al., 1976). These chemicals: (1) have proven lethal and carcinogenic in in vitro bioassays of bacterial and mammalian cells (Ashwood-Smith et al., 1980, 1982; Igali et al., 1970), (2) pose substantial toxicological risks for humans (Scott et al., 1976), and (3) have been recognized as causally related to skin cancer by the World Health Organization (IARC, 1983). The presence of high concentrations of psoralens in other *Apium* spp. and in related genera in the Apiaceae (Umbelliferae) also have been reported (Berenbaum, 1981b; Chaudhary et al., 1986).

Although the linear furanocoumarins were originally thought to be mycotoxins produced by *Sclerotinia*, Beier and Oertli (1983) demonstrated that the phytoalexin response also was initiated by general elictors including copper sulfate, UV light, and cold temperatures. Mechanical damage occurring during harvesting and storage also has been shown to increase concentrations from about 2 ppm to 95 ppm (Chaudhary et al., 1985). In addition, Berenbaum (1981a) and Zangerl and Berenbaum (1987) demonstrated that distribution of psoralens in wild parsnip was significantly correlated with nitrogen content. Thus, induction of psoralens appears to be stress-related while nitrogen levels influence within-plant distribution.

In coastal California, where well over 50% of the celery produced in the United States is grown (Ivey and Johnson, 1986), acidic fogs and ozone are significant stress factors that influence the content and form of nitrogen in plants. Acidic fogs with a pH of 3.6 or lower can cause visible foliar damage and deposit nitrogenous compounds directly on plant surfaces (Shriner, 1986). In the Los Angeles basin, fogs with a pH of 1.69 have been recorded (Hoffman, 1984). However, fogs between pH 2.0 and 3.0 are more common. Although urban encroachment has reduced the total acreage of farmland in Orange and Los Angeles counties, the areas most commonly exposed to high nitrogen acidic fogs, total value of field-grown celery exceeds $5 million dollars (U.S.) annually (Ivey and Johnson, 1986). Atmospheric ozone, which typically reaches concentrations exceeding 0.20 ppm on 20 or more days each year, visibly damages many plant species and significantly reduces total nitrogen content and increases soluble protein levels in tomatoes. Therefore, the purpose of our study was to investigate the psoralen concentrations occurring in a commercial celery variety exposed to stressful atmospheric conditions (ozone and acidic fog) associated with celery production in some areas of coastal California.

METHODS AND MATERIALS

Simulated Acidic Fogs. Simulated acidic fogs were prepared by adjusting distilled water to pH 2.0 and 3.0 with reagent-grade nitric and sulfuric acid mixed at a 2.5 : 1 (v/v) ratio. This acid ratio is typical of fogs in California, but lacked other ionic components of ambient moisture (Waldman et al., 1982). The pH levels also are consistent with fogs occurring near Los Angeles (Hoffman, 1984). Control fogs consisted of distilled water with a pH of 6.3–6.5. Fogs were created within a 1-m^3 chamber using a fogging apparatus designed by Musselman et al. (1985), which operated at 7.03 kg/cm^2 and produced droplets averaging 20 μm in diameter. Temperature at treatment averaged 22–26°C. Shade cloth was used to reproduce incident radiation levels consistent with coastal fogs of no more than 300 $\mu E/m^2/sec$. Three randomly selected celery plants (*Apium graveolens* L., var Tall Utah 52-70) each for control and test fogs were exposed for 4 hr, and then placed in a shaded location to dry for at least 4 hr (22–26°C). These plants were extracted and examined by HPLC (Beier et al., 1983a,b) at 0, 24, and 120 hr posttreatment. This experiment was replicated three times (nine test plants per pH and nine control plants each for 24- and 120-hr measurements, nine untreated plants were analyzed at time 0). To reduce chamber effects, the replicates were run sequentially on three different dates, alternating fogs at pH 2.0, 3.0, and 6.3–6.5. Linear furanocoumarin contents in plants were compared between treatments and controls at 24 and 120 hr postfumigation using Student's *t* tests.

Ozone Treatments. Eight celery plants were exposed to a single 2-hr ozone fumigation at 0.20 ppm in an 0.78-m^3 (interior space) environmental chamber equipped with a fan providing a complete air exchange every 45 sec. Ozone was added after input air was first scrubbed with a charcoal filter. Ozone was generated using an OREC OV35-O ozonator (Ozone Research & Equipment Corp., Phoenix, Arizona) and monitored within the chamber by a model 1008 ozone analyzer (Dasibi Environmental Corp., Glendale, California). Eight control plants also were exposed for 2 hr in the same chamber but without the ozone input. This experiment was replicated three times (24 test plants and 24 control plants). To reduce any chamber effects, the replicates were run sequentially, alternating control and ozone fumigations. Linear furanocoumarin contents in plants were compared between treatments and controls at 24 and 120 hr postfumigation using Student's *t* tests.

Extraction and Quantification. Extraction of the linear furanocoumarins psoralen, bergapten, xanthotoxin, and isopimpinellin was modified from a technique reported by Beier (Beier, 1985; Beier et al., 1983a,b). The frozen plant material was immersed in liquid nitrogen and crushed to a fine powder with a mortar and pestle. The powder was transferred into a 40-ml Ten Broeck homogenizer and further ground in 15 ml of deionized H_2O. The homogenate was

poured into centrifuge tubes, the volume brought up to 25 ml with deionized H_2O, and the samples centrifuged for 6 min at 570*g*. The supernatant was passed into separatory funnels through several layers of cheesecloth and the sediment discarded.

The aqueous phase was extracted once with 20 ml and three times with 10 ml of diethyl ether. Ether fractions were combined, centrifuged at 110*g* to break emulsions, and decanted into a round-bottomed flask. The samples then were reduced to dryness by rotary evaporation. The residue was dissolved in 0.6 ml acetonitrile and 0.4 ml H_2O was added; this flask washing step was repeated four times.

The combined flask rinses were passed through a C18 Sep-Pak cartridge (Waters Associates, Inc., Milford, Massachusetts) which was previously washed with 15 ml MeOH and rinsed with 15 ml H_2O), and the eluate collected. The Sep-Pak cartridge then was washed with 8 ml 60% acetonitrile in H_2O, and the eluates were combined. Fifteen milliliters of anhydrous EtOH was added to the sample to facilitate azeotropic distillation of the water, and the sample subsequently was reduced to approximately 1 ml by rotary evaporation. The remaining water was poured into a test tube and extracted twice with 5 ml ethyl acetate by vigorously shaking the tubes.

After phase separation, the ethyl acetate layers were drawn off with a Pasteur pipete, combined, and passed through a silica Sep-pak cartridge (previously washed with 15 ml of chloroform) to remove polar impurities. The Sep-Pak cartridge was then eluted with 8 ml of 7.5% ethyl acetate in chloroform and the combined eluates were concentrated to dryness by rotary evaporation. The samples were taken up to 1 ml in chloroform and stored at −70°C until analyzed. In all cases, extraction recoveries of standard solutions were equal to or greater than 83%.

Linear furanocoumarins were resolved on a 25-cm Ultrasphere 80 Å pore silica column, preceded by an Ultrasphere 5-μm guard column on a model 334 liquid chromatograph (Beckman, Fullerton, California). The solvent system consisted of HPLC grade chloroform (Aldrich, Milwaukee, Wisconsin) pumped at a flow rate 1.5 ml/min (modified from Beier, 1985). Eluting components were monitored with a Beckman 160 UV detector set at 254 nm; sample peaks were recorded and integrated on a model 3392A integrator (Hewlett Packard, Avondale, Pennsylvania). Recovery of standards always exceeded 83%. Elutants were identified using mass spectrometry performed on a 7070E mass spectrometer (VG Analytical, Ltd., Wythenshawe, U.K.). Samples were analyzed using a solid probe (200–250°C) under electron ionization conditions (70 eV, source temperature = 150°C). A mass range of 100–300 daltons was scanned. The standards used in this study were psoralen, bergapten (5-methoxypsoralen), and xanthotoxin (8-methoxypsoralen), and isopimpinellin (5,8-methoxypsoralen) (Figure 1). The first three were purchased from Aldrich, and the last was

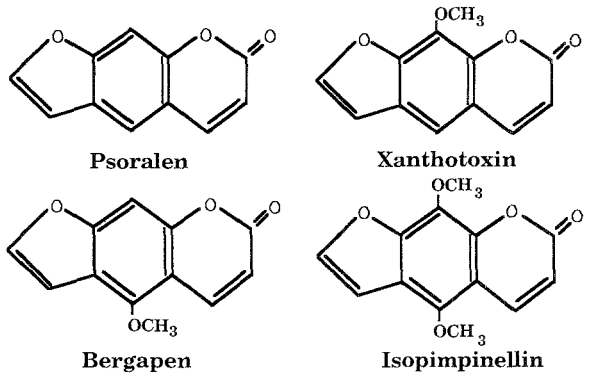

FIG. 1. Structures of the four linear furanocoumarins found in celery.

kindly provided by Dr. R.C. Beier of the Southern Plains Area Veterinary Toxicology and Entomology Research Laboratory in College Station, Texas.

RESULTS AND DISCUSSION

Mass Spectrometry. The most important fragment ions for psoralen included: *m/z* 187 (24%); 186 (71%, $[M]^+ \cdot$); 159 (22%); 158 (100%); 130 (23%); 129 (45%); 103 (11%); 102 (53%); and 101 (27%). Key fragment ions for bergapten *m/z* 217 (26%); *m/z* 216 (72%, $[M]^+ \cdot$); 201 (42%); 188 (27%); 187 (19%); 174 (22%); 173 (94%); 146 (21%); 145 (74%); 144 (20%); 102 (22%); and 100 (19%). Identifying fragment ions for xanthotoxin included: *m/z* 217 (26%); *m/z* 216 (72%, $[M]^+ \cdot$); 201 (60%); 188 (45%); 174 (40%); 173 (100%); 158 (22%); 145 (92%); 129 (37%); 117 (21%); 116 (23%); 102 (25%); and 101 (21%). Key fragment ions for isopimpinellin included: *m/z* 246 (39%, $[M]^+ \cdot$); 231 (100%); 203 (27%); 188 (39%); 175 (33%); 160 (30%); 147 (25%); 143 (23%); 104 (49%); and 103 (21%). No other linear furanocoumarins were detected.

Simulated Acidic Fogs. Leaves from plants exposed to acidic fogs with a pH of 2.0 had significantly higher concentrations of the phototoxic furanocoumarins than plants treated with control fogs within 24 hr posttreatment (Figure 1). At 120 hr after exposure, all linear furanocoumarins reached significantly higher levels after exposure to 2.0 pH fogs. In contrast, fogs of 3.0 pH did not increase the levels of any furanocoumarins at 24 hr. However, all compounds except psoralen increased significantly in leaves at 120 hr. Foliar and petiole lesions were observed on all plants exposed to fogs with a pH of 2.0, but not on plants treated with control fogs or with fogs of pH 3.0.

Leaves consistently had higher concentrations than petioles and the linear

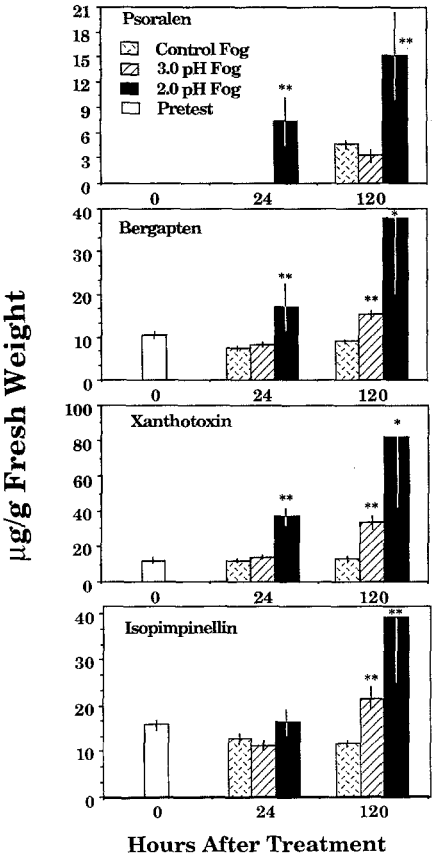

FIG. 2. Acute impact of acidic fogs of pH 2.0 and 3.0 and control fogs (pH 6.3–6.5) on linear furanocoumarin content in celery leaves. Bars delineate standard errors. Asterisk(s) next to points indicate significant differences from plants treated with a control fog (*$P \leq 0.05$, **$P \leq 0.01$, Student's t test, 16 df).

furanocoumarins psoralen, bergapten, xanthotoxin, and isopimpinellin (Figures 1 and 2). Although this trend has been reported previously, the levels of psoralen, bergapten, and xanthotoxin (phototoxic furanocoumarins) from control plants in our test were sixfold higher in leaves and stems than observed by Beier et al. (1983a) (mean from leaves or petioles for psoralen ≤ 0.15, bergapten ≤ 1.5, xanthotoxin 3.5 ppm), but were similar (ca. 1.4-fold greater) to the concentrations reported by Berkley et al. (1986) (phototoxic furanocoumarins = 11.2 ppm in petioles and = 20.4 ppm in leaves). Such increases may be due

to varietal effects (Berkley et al., 1986), but direct comparisons with previous studies were not possible because the cultivars tested were not reported.

The foliar concentrations of the phototoxic furanocoumarins in our study are of considerable concern because of a new trend in marketing intact celery plants (leaves not trimmed) (T. Batkin, manager, California Celery Research Advisory Board, Dinuba, California, personal communication) rather than the more common "topped" celery (most leaves removed). Even in our control plant foliage, levels of the phototoxic furanocoumarins were more than twice (ca. 30 μg/g fresh weight) the critical level of 18 μg/g fresh weight known to cause phototoxic dermatitis (Austad and Kavli, 1983). Foliage from plants exposed to an acidic fog with a pH of 3.0 exceeded 50 μg/g fresh weight. The foliar concentrations of these compounds in plants exposed to a single fog of pH 2.0 reached 140 μg/g fresh weight and should be considered hazardous.

The concentrations of phototoxic furanocoumarins in petioles (59.7 μg/g fresh weight) of plants exposed to fogs of pH 2.0 exceeded the critical level of 18 μg/g by over 300%. Thus, even celery marketed with the leaves trimmed may have considerable potential for human health concerns if exposed to highly acidic fogs. However, petioles from plants treated with a control fogs or a fog with a pH of 3.0 contained concentrations of these chemicals below the critical level.

Ozone Fumigations. In general, ozone fumigation had a markedly different effect on content of linear furanocoumarins than did acidic fogs. Ozone fumigation was conducted at 20 ppm, a level which is reached in excess of 20 or more days each year in agricultural areas near Los Angeles. Foliage of plants exposed to ozone had significantly reduced concentrations of psoralen, bergapten, and isopimpinellin; xanthotoxin levels were not significantly reduced (Figure 3). At 120 hr posttreatment, no significant differences were evident between treatments. Levels of these compounds in petioles at 24 hr followed similar trends but were not significant at the $P < 0.05$ level (Figure 4). No differences in concentration were detected at 120 hr following ozone fumigation. Several mechanisms for the observed reductions in furanocoumarin concentrations appear possible: (1) the ozonolysis of the olefinic bonds in the furan or lactone rings found on these compounds, (2) the induction of degradation enzymes is inhibited, and/or (3) the inhibition of biosynthetic enzymes is occurring. Additional research will be required to document the critical mechanism(s).

Although the plants examined in the acidic fog and ozone trials were all from the same seedlot, planted at the same time, and agronomically treated the same, the control plants in the ozone tests developed consistently higher quantities of the linear furanocoumarins. This could have been caused by a reduction in concentration following control fogs, or more likely, an increase in furanocoumarin content occurring as a result of plant size (Austad and Kavli, 1983).

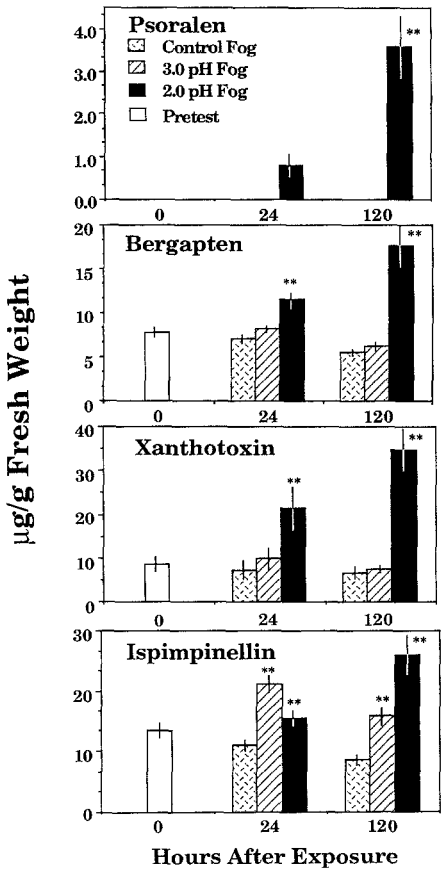

FIG. 3. Acute impact of acidic fogs of pH 2.0 and 3.0 and control fogs (pH 6.3–6.5) on linear furanocoumarin content in celery petioles. Bars delineate standard errors. Asterisk(s) next to points indicate significant differences from plants treated with a control fog (*$P \leq 0.05$, **$P \leq 0.01$, Student's t test, 16 df).

The plants used in the ozone trial were about eight weeks older than those used in the acidic fog tests and had reached the 36/carton stage. Nonetheless, the foliar contents of these chemicals were above the levels required to cause contact dermatitis. Because contact dermatitis in celery handlers is common [19 cases in 48 handlers in the most recent study (Seligman et al., 1987)], we suspect that the concentrations of phototoxic furanocoumarins found in our study are not unusual. At the least, the effects of atmospheric pollutants will help

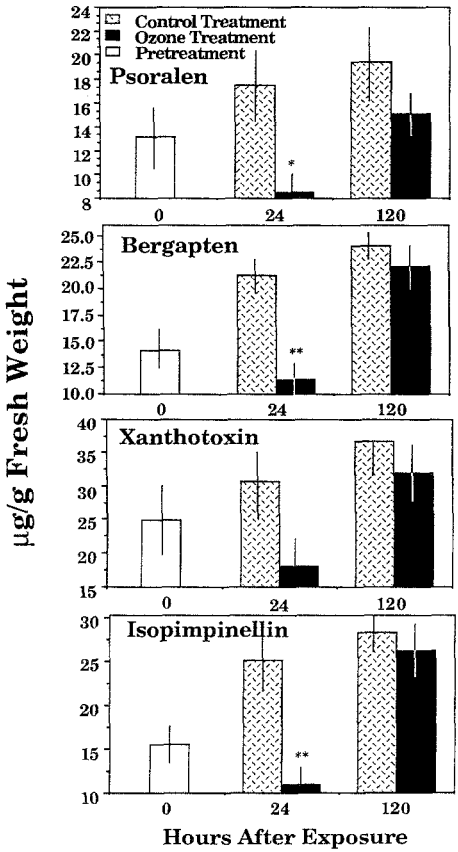

FIG. 4. Acute effects of 2-hr ozone fumigation of celery at 20 ppm on linear furanocoumarin content in foliage of a commercial celery variety. Bars delineate standard errors. Asterisk(s) next to points indicate significant differences from plants not treated with ozone (*$P \leq 0.05$, **$P \leq 0.01$, Student's t test, 16 df).

account for some of the variability in furanocoumarin contents reported in the literature.

The concentrations of linear furanocoumarins found in our study may be conservative. None of the plants used in any portion of this study exhibited symptoms of disease or were subjected to mechanical damage or cold temperatures, which could increase furanocoumarin content (Austad and Kavli, 1983; Ashwood-Smith et al., 1985; Beier and Oertli, 1983; Chaudhary et al., 1985). In addition, plants in our study were subjected to only acute exposure to atmo-

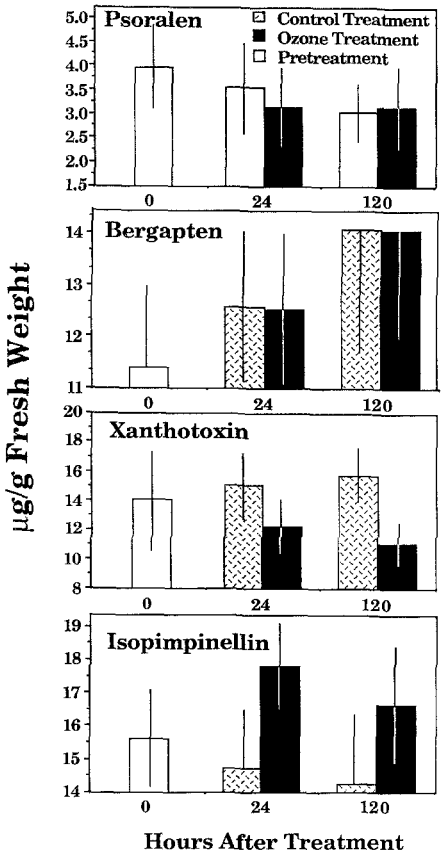

FIG. 5. Acute effects of 2-hr ozone fumigation of celery at 20 ppm on linear furanocoumarin content in petioles of a commercial celery variety. Bars delineate standard errors. Asterisk(s) next to points indicate significant differences from plants not treated with ozone ($*P \leq 0.05$, $**P \leq 0.01$, Student's t test, 16 df).

spheric pollutants. The impact of longer or chronic exposures to acidic fogs or ozone in combination with other environmental stresses on the production of linear furanocoumarins is worthy of additional study. To our knowledge this is the first report of anthropogenic air pollutants inducing production of plant metabolites which can be injurious to human health. These results raise the intriguing possibility that continuing production of atmospheric air pollutants could be having deleterious effects on our food supply beyond simply decreasing yields (Leung et al., 1982) or increasing insect damage (Trumble et al., 1987). This, in turn, leads to the possibility that certain geographic areas subject

to acidic fogs may be less desirable for production of celery for human consumption.

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INHIBITION OF CUCUMBER LEAF EXPANSION BY FERULIC ACID IN SPLIT-ROOT EXPERIMENTS[1]

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Abstract—Experiments were conducted to determine how the proportion of a root system in contact with an allelopathic compound may affect seedling responses. Cucumber seedlings grown in a split-root nutrient culture system were given either single (1 mM) or multiple applications (0.5 mM) of ferulic acid. Seedlings receiving single applications were left in the treatment solutions for two days and then harvested, while seedlings receiving multiple applications had their solutions changed every other day for a total of three changes. Leaf areas were determined daily starting with the initial ferulic acid treatment. Mean absolute and mean relative rates of leaf expansion were inversely related to the proportion of the root system in ferulic acid solution. Leaf expansion was inhibited primarily during the first 24 hr after each treatment. A partial recovery of growth occurred during the second 24-hr period following each treatment. Root length was reduced by ferulic acid. These results suggest that information on root and allelochemical distribution in soils is important when assessing the potential of allelopathic interactions between plants.

Key Words—Allelopathy, ferulic acid, cucumber seedlings, *Cucumis sativus*, split-root treatments, leaf expansion, mean absolute growth rate, mean relative growth rate, growth inhibition.

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INTRODUCTION

Many allelopathic compounds have been identified in soils that may affect the growth and development of plants (Whitehead, 1964; Wang et al., 1967; Whitehead et al., 1982). These are derived primarily from organic debris and living roots, which are normally unevenly distributed on or in the soil. Thus, for any given point in time, only a portion of a root system may be in contact with allelopathic compounds, and the proportion of the root system in contact with such agents will vary with time as the roots grow in the soil. Most bioassay studies, however, treat the whole root system (Patterson, 1981; Rice, 1984; Blum and Dalton, 1985). How plant growth may be affected by the proportion of the root system in contact with allelopathic agents has not been investigated. Several authors, however, have suggested that this may substantially affect plant response (Patrick et al., 1964; Lynch, 1985; Blum and Rebbeck, 1989).

The objective of this investigation, therefore, was to determine how seedling responses to allelopathic agents may be modified by varying the proportion of the root system in contact with an allelochemical. We chose ferulic acid and cucumber seedlings (*Cucumis sativus* cv. Early Green Cluster) for this study. Ferulic acid has been identified as an allelopathic compound (Wang et al., 1967; Rasmussen and Einhellig, 1977; Patterson, 1981) that affects cucumber seedling growth in a predictable manner (Blum and Dalton, 1985).

METHODS AND MATERIALS

A nutrient culture system was chosen for these experiments because contact between ferulic acid and roots can be easily controlled and every molecule in the solution in which the roots are immersed has the potential to interact with the roots. In soil systems both roots and ferulic acid are subject to uneven distribution. Absolute and relative growth rates were used because they provided a sensitive measure of ferulic acid effects on cucumber seedlings. In addition, Blum and Dalton (1985) observed that leaf area of cucumber seedlings and dry weight were directly related.

Single Ferulic Acid Treatment. Cucumber seeds (*Cucumis sativus* cv. Early Green Cluster) were placed in trays containing vermiculite and Hoagland's solution (pH 5.6) (Hoagland and Arnon, 1950). The seeds were allowed to germinate in the dark at 25°C for four days. On the fourth day, emerging seedlings were exposed to light for 8 hr before being transferred to containers with 110 ml of Hoagland's solution. The seedlings were suspended by foam collars through the lids of the containers. The plants were grown for an additional eight days under light banks (twelve 40-W cool white fluorescent and six 25-W incandescent bulbs). Seedlings received 140 $\mu E/m^2/sec$ over a 12-hr photoperiod

(Blum and Dalton, 1985). The growth temperatures ranged from 21 to 30°C. Water lost via evapotranspiration was replaced daily with deionized water. Nutrients supplied by the 110 ml were adequate since Blum and Dalton (1985) had previously demonstrated that 110 ml of 1/4 to 1/2 strength Hoagland's nutrient solution supplied adequate nutrition for cucumber seedlings over a 17-day growth period under these light banks. On day 12 the seedlings were suspended by foam collars through lids of 700-ml containers ($N = 25$). The seedling root systems were suspended into smaller containers within the 700-ml containers. The smaller containers were filled with 110 ml of Hoagland's solution (CONT) or a 1 mM ferulic acid–Hoagland's solution (FA). Containers were covered with aluminum foil to exclude light. Deionized water was added daily to each container to compensate for evapotranspiration. The initial pH of all solutions was 5.6.

Root systems of seedlings were placed in either a single container (100% CONT or 100% FA) or split between CONT and FA (~25, 50, or 75% FA) containers. The primary root was placed in the container with the largest root mass. In cases of 50:50 split, the primary root was randomly placed in either CONT or FA. For each seedling, the percentage of root length in each container was calculated based on root length measurements (see data analysis below). Only one container was used for the 100% CONT (controls) and 100% FA treatments since the Hoagland's solution supplied was more than adequate for normal growth of cucumber seedlings (Blum and Dalton, 1985).

Length and width measurements (mm) of the leaves on each plant were recorded daily starting on day 12. On day 14, roots were severed from the shoots and placed into 70% ethanol until they could be measured. The CONT and FA treated roots were stored separately. To measure total root length, all secondary lateral roots were severed from the primary root, and the primary and secondary roots were measured to the nearest centimeter. Root systems of five seedlings were also harvested on day 12 to provide an estimate of the average starting root length.

Multiple Ferulic Acid Treatments. The general procedures and growth conditions of this experiment were the same as the previous experiment. In this case, however, roots were treated three times with 0.5 mM ferulic acid solution ($N = 25$). This lower concentration was used because the 100% FA (1.0 mM) plants in the first experiment wilted.

Seedlings were treated on days 13, 15, and 17 by replacing all solutions. Leaf areas were measured daily starting with day 13 and ending with day 19, when the seedlings were harvested. In addition, sets of five seedlings with 100% of their root systems in CONT or FA were harvested on days 13 (CONT only), 15, 17, and 19.

Data Analyses. For both experiments, the daily leaf area (cm^2) of each seedling was determined from length (L) and width (W) measurements (mm)

of each leaf using the following formula: leaf area = $-1.457 + [0.00769 * (L * W)]$ (Blum and Dalton, 1985). Mean absolute and mean relative rates of leaf expansion (AGR and RGR, respectively) were then calculated (see Radford, 1967) by the following equations:

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

and

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

where AGR is in cm^2/day and RGR is in cm^2/cm^2/day.

Root growth of control plants (100% CONT) was greater, on average, than root growth of plants in ferulic acid (100% FA). This suggested that for the split-root systems the proportion of roots in ferulic acid changed with time. To adjust the proportions, we assumed (1) that roots of the split-root system in CONT and in FA grew independently and (2) that root growth in the 100% CONT or FA was representative of the corresponding root portions of the split-root system.

Average root lengths for the 100% CONT or FA treatments (roots were harvested on days 12 and 14 of the first experiment and days 13, 15, 17, and 19 of the second experiment) were used to calculate appropriate coefficients for each time interval. The coefficients were calculated, for example, by taking the average root length for day 17 (a) and 19 (b) and solving for x (where $x = a/b$). These coefficients in conjunction with final root lengths (day 14 or 19) were used to back-calculate the ratio of roots in FA and CONT for the split-root seedlings on day 12 for experiment 1 and days 13, 15, and 17 for experiment 2. Data were analyzed using the Statistical Analysis System (SAS) programs for linear and multiple regressions (SAS Institute Inc., 1985).

RESULTS

Single Ferulic Acid Treatment. As the proportion of roots of cucumber seedlings treated with 1 mM ferulic acid increased, absolute (AGR) and relative (RGR) growth rates declined (Figure 1). When 100% of the root system of these seedlings was placed in FA, they wilted shortly after treatment but became turgid again within 24 hr. Leaf area data for these seedlings were deleted from the analysis since there was such an abrupt change in growth rates because of wilting. We felt, however, that root adjustments could be based on these seedlings because all roots in the 1 mM ferulic acid would be similarly affected. The slopes of the regression lines for both the AGR and RGR (slopes for growth periods 12–13 and 13–14 were -0.0947, -0.0470 for the AGR and -0.0024,

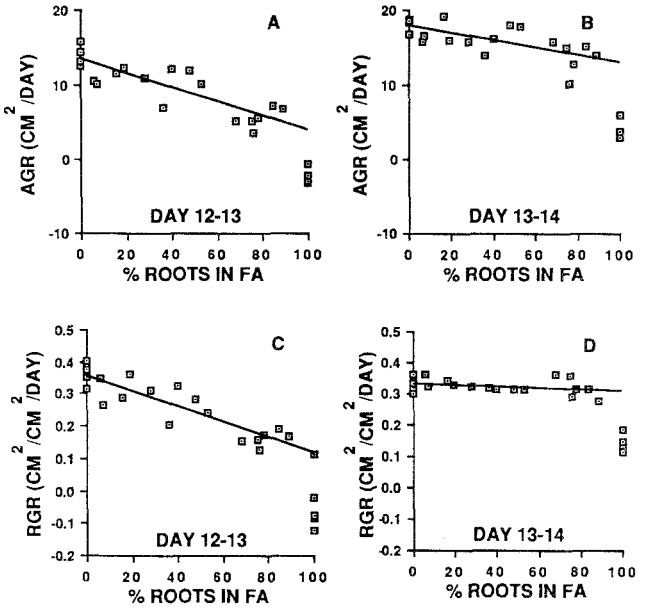

FIG. 1. Mean absolute (AGR) and mean relative (RGR) rates of leaf expansion of cucumber seedlings for which various proportions of roots (% ROOTS IN FA) were treated with 1 mM ferulic acid on day 12. R^2 values for A, B, C, and D were 0.86, 0.81, 0.85, and 0.76, respectively.

−0.00026 for the RGR) shifted significantly during the second 24-hr period after treatment, indicating a recovery of leaf expansion for the treated seedlings (Figure 1). The mean pretreatment root length for both CONT and FA ± standard error (SE) on day 12 was 157.76 ± 13.7 cm. The mean root lengths ± SE for the two treatments on day 14 were 203.9 ± 11.6 and 309.1 ± 17.3 for FA and CONT, respectively.

Multiple Ferulic Acid Treatments. The leaf area of seedlings for which the entire root system (100% FA) was treated with ferulic acid was significantly less than the controls (100% CONT) one day after the second treatment (day 16; Figure 2A), while a significant reduction in the rate of leaf expansion was observed 24 hr after the first treatment (i.e., growth period 13–14; Figure 3). All subsequent ferulic acid treatments reduced leaf area and leaf expansion during the first 24-hr period after treatment. Recovery of growth rates was evident during the second 24-hr period after each 0.5 mM ferulic acid treatment (Figure 3). This was particularly evident for the RGR. A significant reduction in mean

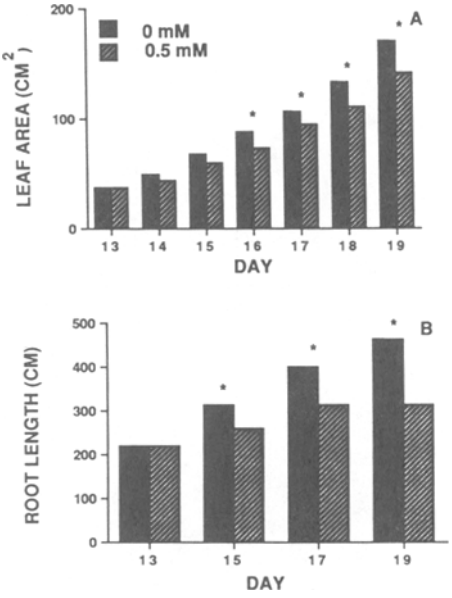

FIG. 2. Mean leaf area and root length of cucumber seedlings treated (100% of the roots in CONT or FA) with 0 or 0.5 mM ferulic acid solution on days 13, 15, and 17. Asterisks indicate significance at 0.05 levels.

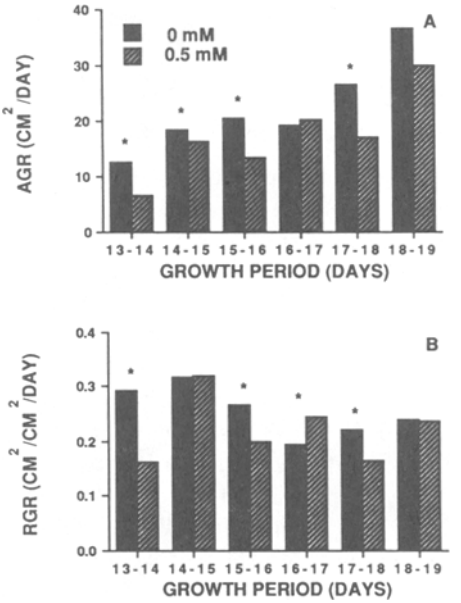

FIG. 3. Mean absolute (AGR) and mean relative (RGR) rates of leaf expansion of cucumber seedlings treated (100% of the roots in CONT or FA) with 0 or 0.5 mM ferulic acid solution on days 13, 15, and 17. Asterisks indicate significance at 0.05 level.

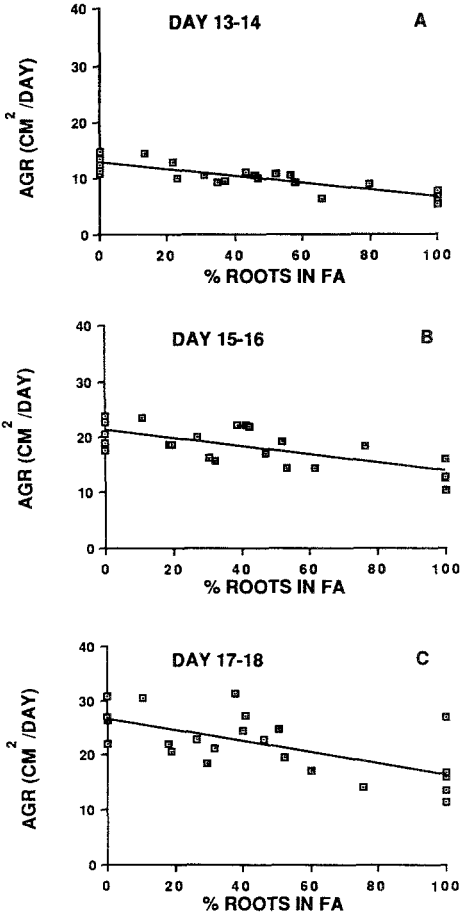

FIG. 4. Absolute (AGR) rates of leaf expansion of cucumber seedlings for which various proportions of roots (% ROOTS IN FA) were treated with 0.5 mM ferulic acid on days 13, 15, and 17. R^2 values for A, B, and C were 0.74, 0.50, and 0.42, respectively.

root length was observed when 100% FA roots were compared to 100% CONT roots 48 hr after each treatment (Figure 2B).

As the proportion of the root system treated with 0.5 mM ferulic acid increased, the growth rates of leaves declined (Figures 4 and 5). The best models were generated for the 13 to 14-day growth period. The scatter of the data along the regression lines increased for the subsequent growth periods. R^2 values for the AGR declined from 0.74 (day 13–14) to 0.42 (day 17–18) and the R^2 values for the RGR declined from 0.79 to 0.35.

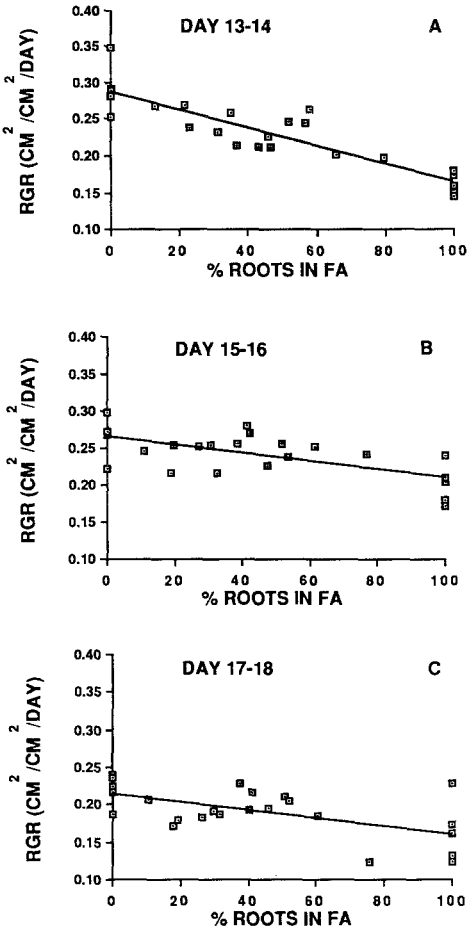

FIG. 5. Mean relative (RGR) rates of leaf expansion of cucumber seedlings for which various proportions of roots (% ROOTS IN FA) were treated with 0.5 mM ferulic acid solution on days 13, 15, and 17. R^2 values for A, B, and C were 0.79, 0.41, and 0.35, respectively.

DISCUSSION

The transitory effects of ferulic acid were apparent from the initial reduction of leaf expansion during the first 24-hr period and the observed recovery of leaf expansion during the second 24-hr period after each treatment. Similar transitory effects of ferulic acid were observed by Blum and Rebbeck (1989). They noted that inhibitory effects of ferulic acid on cucumber seedling growth

required the continued presence of the acid in the nutrient solution. Once ferulic acid was removed from the nutrient solution, "normal" root and leaf growth resumed.

Leaf expansion (i.e., AGR, RGR) was inversely related to the proportion of the root system in ferulic acid solution for both the 1.0 and 0.5 mM ferulic acid treatments. This is the first experimental evidence that the proportion of a plant root system in contact with a known allelopathic compound may indeed affect the level of growth inhibition. These observations, if confirmed for other species and other allelopathic compounds, suggest that a knowledge of the concentration and distribution of allelopathic compounds in the rhizosphere of a plant may be more meaningful in determining potential allelopathic interactions between plants than concentrations and distributions determined from bulk soil samples.

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ATTRACTION OF *Oryzaephilus surinamensis* (L.) AND *Oryzaephilus mercator* (FAUVEL) (COLEOPTERA: CUCUJIDAE) TO SOME COMMON VOLATILES OF FOOD[1]

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Abstract—Responses by adult *Oryzaephilus surinamensis* (L.) and *Oryzaephilus mercator* (Fauvel) to various food volatiles were assessed by means of a two-choice, pitfall olfactometer. The individual experimental stimuli, all potential products of lipid oxidation, had a range of attractive doses of $\leq$ 1000-fold over the test dose ranges of 0.001–100 μg, or 0.01–1000 μg. Of 13 aliphatic C_3–C_{14} aldehydes and benzaldehyde tested for *Oryzaephilus* spp., 10 C_3–C_{10} aliphatic aldehydes and benzaldehyde showed some attractiveness for both species. For *O. mercator*, nonanal had the lowest lower threshold for positive response at 0.01 μg. The addition of small amounts of nonanal or of a 1 : 1 : 1 mixture of hexanal, octanal, and nonanal to small amounts of cucujolide aggregation pheromones enhanced response by mixed-sex *O. mercator* to the pheromones. Eleven aliphatic C_2–C_9 free fatty acids showed some attractiveness for both *Oryzaephilus* spp. Isovaleric acid and valeric acid had the lowest lower thresholds for positive response at 0.1 μg for *O. mercator* and *O. surinamensis*, respectively. Four olefinic oat volatiles were found to possess various degrees of attractiveness for both *Oryzaephilus* spp. The data suggest that food volatiles in this study might be used by *Oryzaephilus* spp. as host-finding kairomones in nature.

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Key Words—*Oryzaephilus surinamensis* (L.), *Oryzaephilus mercator* (Fauvel), Coleoptera, Cucujidae, attractants, aliphatic C_3–C_{14} aldehydes, benzaldehyde, aliphatic C_2–C_9 free fatty acids, 2(*E*),4(*E*)-heptadienal, 2(*E*),4(*E*)-nonadienal, 3(*E*),5(*E*)-octadien-2-ol, 3(*E*),5(*E*)-octadien-2-one, oat volatiles.

INTRODUCTION

The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), and the merchant grain beetle, *O. mercator* (Fauvel), are cosmopolitan pests of stored products. *O. surinamensis* is one of the most common pests of stored grain and processed cereals in Canada, United States, Britain, Australia, Asia, Africa, and South America (Sinha and Watters, 1985). *O. mercator* has become firmly established as a household pest in North America, especially on cereal products (Loschiavo and Sabourin, 1982).

Identification of attractive semiochemicals for *Oryzaephilus* spp. could contribute to the development of integrated control programs (Levinson and Levinson, 1979; Burkholder, 1981). Feeding males of both *Oryzaephilus* spp. produce macrolide aggregation pheromones, a class of pheromones given the trivial name cucujolide because of its prevalence in the family Cucujidae (Oehlschlager et al., 1988). These cucujolides have been identified as 3(*Z*),11(*R*)-dodecen-11-olide [(*R*)-II] and 3(*Z*),6(*Z*),11(*R*)-dodecadien-11-olide [(*R*)-IV] for *O. mercator*, and (*R*)-IV, 3(*Z*),6(*Z*)-dodecadienolide (IX), and 5(*Z*),8(*Z*),13(*R*)-tetradecadien-13-olide [(*R*)-V] for *O. surinamensis* (Pierce et al., 1985, 1987). In addition, males and females of both *Oryzaephilus* spp. maintained at low population densities produce another aggregation pheromone, (*R*)-(−)-1-octen-3-ol (Pierce et al., 1989).

Pierce et al. (1981) reported that *Oryzaephilus* spp. were attracted to Porapak Q-trapped volatiles from rolled oats or brewer's yeast. Unpublished analyses of these volatiles by coupled gas chromatography–mass spectroscopy (GC-MS) revealed the presence of hexanal, heptanal, octanal, nonanal, decanal, dodecanal, and benzaldehyde in the rolled oat volatiles, while benzaldehyde and isobutyric, 2-methylbutyric, isovaleric, and valeric acids were detected in brewer's yeast volatiles. Hexanal and nonanal comprised ~10% and ~14%, respectively, of the Porapak Q-trapped volatiles from rolled oats. Mikolajczak et al. (1984) have identified a number of volatile attractants from rolled oats for *O. surinamensis*. Additionally, some attractants from carobs also have been identified for *O. surinamensis* by O'Donnell et al. (1983) and Stubbs et al. (1985).

Our current objective was to screen various host-produced volatiles (primarily aldehydes and free fatty acids) as potential attractants for both *Oryzae-*

philus spp. Such attractants could be candidates for the formulation of a more cost-effective field bait containing host volatiles as well as cucujolides.

METHODS AND MATERIALS

Experimental Insects. O. mercator and *O. surinamensis* were reared on large-flake rolled oats and brewer's yeast (95:5, w/w) in 3.8-liter glass jars in an environmental chamber maintained at 28–30°C and 40–60% relative humidity in darkness.

Experimental Stimuli for Bioassay. Fatty acids, aldehydes, 2(*E*),4(*E*)-heptadienal, and 2(*E*),4(*E*)-nonadienal were purchased from commercial supply houses, and all but the fatty acids were distilled before use. Compounds were checked by gas chromatography (GC) before use and found to be ≥99% pure. Macrocyclic lactones for bioassay were racemic cucujolide II (Oehlschlager et al., 1983) and racemic cucujolide IV (Millar and Oehlschlager, 1984). Addition of methyllithium in ether to an etheral solution of freshly distilled 2(*E*),4(*E*)-heptadienal (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) gave, after aqueous work-up and bulb-to-bulb distillation, 3(*E*),5(*E*)-octadien-2-ol in 88% yield, which was 95% pure by GC analysis. Oxidation of 3(*E*),5(*E*)-octadien-2-ol with active MnO_2 in pentane gave, after removal of MnO_2 by filtration, evaporation of solvent, and bulb-to-bulb distillation, 3(*E*),5(*E*)-octadien-2-one in 81% yield, which was 95% pure by GC analysis. Analyses of the dienol and dienone by GC-MS indicated that the impurity in each was an isomer of unknown double-bond configuration.

Instrumental Methods. A Hewlett-Packard 5830A gas chromatograph equipped with a 18835B capillary inlet system and a flame-ionization detector was employed for analyses by GC. Helium was the carrier gas, and the injection port and detector temperatures were 260°C and 270°C, respectively. Samples were analyzed on an open-tubular glass column (40 m × 0.5 mm ID) coated with SP-1000 (Supelco Canada Ltd., Oakville, Ontario, Canada). The temperature program for analytical GC was 70°C for 2 min, then 4°C/min to 180°C, holding for 30 min or less.

GC-MS was performed on a Hewlett-Packard 5895B GC-MS-data system fitted with a fused silica column (30 m × 0.25 mm ID) coated with Carbowax 20 M (J&W Scientific, Folsom, California) with helium as the carrier gas.

Bioassay Procedures. Bioassays were conducted at 23°C and 60% relative humidity using mixed-sex beetles 5–12 weeks posteclosion. To ensure a uniform state of preconditioning for *O. mercator*, each replicate of 12 beetles was held in a 60-ml glass vial without food for 20 hr at 23°C darkness prior to a bioassay (Borden et al., 1979). To obtain maximum responsiveness from *O.*

surinamensis, up to 1500 beetles were preconditioned without food for 48 hr at 23°C in darkness in a 6-liter Erlenmeyer flask, through which charcoal-filtered, humidified air was drawn at 1.5 liters/min (Pierce et al., 1985). Since response by *O. surinamensis* to olfactory stimuli is extremely sensitive to population density (Pierce et al., 1983), test beetles were maintained at a low density of 1000 beetles/kg medium for at least one week before bioassays commenced.

A two-choice, pitfall olfactometer (Pierce et al., 1981) was utilized to test attractiveness of experimental stimuli. Filter paper disks, treated with a 10-μl aliquot of either an experimental stimulus in purified pentane or purified pentane as a control, were put singly into the bottoms of two glass vials suspended from holes in the bottom of a plastic Petri dish arena. (All test compounds dissolved completely in pentane at the concentrations used.) Twelve beetles were released into the dish, and the lid was replaced. Bioassays for each test solution were replicated 12 times (i.e., 12 olfactometers), using 12 fresh beetles in each replicate. After 2 hr in darkness, the numbers of beetles in experimental and control vials were recorded. For the compounds in Tables 1, 3, and 4 (below), all doses for a particular test compound were done in the same bioassay session, beginning with 12 replicates of the lowest dose and proceeding in sequence to the highest dose. The responsiveness of the test beetles was checked by bioassaying a standard low dose of cucujolides at the start of a bioassay session.

The untransformed data were analyzed by the paired-sample t test (Zar, 1984). For some experiments, the data also were analyzed by ANOVA followed by the Newman-Keuls test. Results were expressed as mean percent response = 100 (experimental − control)/N where experimental and control were the number of beetles in the vials containing the experimental and control disks, respectively, and N was the total number of insects released into the dishes. For each test solution, the number of responding insects was $\geq 90\%$ of those released.

RESULTS

For *O. mercator*, nonanal was the aldehyde having the lowest threshold for positive response, with significant positive responses demonstrated at experimental doses ranging from 0.01 to 10 μg (Table 1). Hexanal, octanal, 2-methylpropanal, and benzaldehyde had the next lowest thresholds for positive response at 0.1 μg. Over the test dose range examined, nonanal, hexanal, and 2-methylpropanal had the greatest attractive ranges of 1000-fold each.

For *O. surinamensis*, hexanal, pentanal, 2-methylpropanal, and nonanal were the aldehydes having the lowest thresholds for positive response at the

TABLE 1. RESPONSE BY *O. mercator* AND *O. surinamensis* IN TWO-CHOICE, PITFALL BIOASSAY TO HOST-PRODUCED ALDEHYDES

Test species	Aldehyde tested	$\bar{X}$% response[a]					
		0.001 μg	0.01 μg	0.1 μg	1 μg	10 μg	100 μg
O. mercator	Propanal	8.3	1.4	5.6	45.9***	63.9***	5.5
	2-Methylpropanal	11.1	−5.6	19.4*	37.5**	36.0***	44.4***
	Butanal	12.5	−8.3	−6.9	12.5	23.6*	43.0***
	3-Methylbutanal	−1.2	3.1	2.8	15.3	38.9***	62.5***
	Pentanal	8.3	1.4	12.5	41.7***	55.6***	50.0***
	Hexanal	9.0	18.1	27.6**	40.3***	46.6***	33.3**
	Heptanal	−1.4	15.3	17.4	34.5***	39.6***	2.8
	Octanal	7.6	7.6	29.8**	43.1***	39.6***	−2.0
	Nonanal	6.2	30.6**	43.8***	56.8***	38.9***	11.1
	Decanal	−3.4	5.5	2.8	21.5**	50.0***	−15.3
	Benzaldehyde	6.2	13.9	22.8*	33.3***	41.7***	−7.0
O. surinamensis	Propanal	1.9	−2.1	−7.7	0.7	13.5	20.9*
	2-Methylpropanal	−5.6	15.9	23.9*	24.3**	29.7**	58.3***
	Butanal	−3.0	13.2	4.3	3.6	12.1	33.8***
	3-Methylbutanal	4.2	2.8	6.8	24.4*	26.7**	44.0***
	Pentanal	7.8	−0.8	25.6*	27.1**	58.2***	48.9***
	Hexanal	12.5	15.9	27.4**	47.6***	46.2***	−9.7
	Heptanal	11.4	14.5	20.3	43.7***	45.7***	24.8*
	Octanal	4.2	9.7	16.0	22.9*	33.8**	12.7
	Nonanal	6.9	18.1	24.4*	31.0**	27.1**	9.2
	Decanal	−8.4	16.7	20.9	25.7*	33.3***	17.7
	Benzaldehyde	4.2	0.7	5.8	17.2*	35.2**	11.3

[a] Significant response (t test) to experimental stimulus indicated by: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. $N = 12$ replicates per treatment, 12 adults per replicate.

0.1-μg dose (Table 1). Over the test dose range examined, pentanal and 2-methylpropanal had the greatest attractive ranges of 1000-fold each. Neither species responded to undecanal, dodecanal, or tetradecanal over an experimental dose range of 0.001–100 μg (data not shown).

When combined in a 1 : 1 : 1 mixture, three of the more attractive aldehydes for *O. mercator*, hexanal, octanal, and nonanal, induced attractive responses over a 0.01- to 100-μg dose range (Table 2). When low amounts (0.002 or 0.02 μg) of cucujolides II and IV, response to the aggregation pheromones by mixed-sex *O. mercator* was enhanced.

For both species, thresholds for positive response to free fatty acids were relatively high (Table 3). For *O. mercator*, isovaleric acid had the lowest threshold at 0.1 μg, and isovaleric and heptanoic acids gave the greatest attrac-

TABLE 2. RESPONSE BY *O. mercator* IN TWO-CHOICE, PITFALL BIOASSAY TO RACEMIC CUCUJOLIDES II + IV, ALDEHYDES, AND MIXTURES OF II + IV AND ALDEHYDES

	Dosage (μg)			
Exp.[a]	II + IV (1:1)	Nonanal	Hexanal + octanal + nonanal (1:1:1)	$\bar{X}$% response[b]
1			0.001	10.1
			0.01	24.8*
			0.1	49.9***
			1	66.9***
			10	54.2***
			100	30.6**
2	0.002			22.5*a
		0.01		25.5**a
	0.002	0.01		56.5***b
			0.01	21.9*a
	0.002		0.01	45.3***b
3	0.02			36.1**a
		0.1		41.1***ab
	0.02	0.1		49.0***b
			0.1	45.6***ab
	0.02		0.1	66.8***c

[a] Each experiment was completed in a separate 2-hr session.
[b] Significant response (t test) to experimental stimulus indicated by: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $N = 12$ replicates per treatment, 12 adults per replicate. Means within experiments 2 or 3 followed by the same letter not significantly different (Newman-Keuls test, $P < 0.05$).

tive ranges of 1000-fold each over the test dose range examined. Valeric acid induced the lowest threshold for positive response by *O. surinamensis* at 0.1 μg; and acetic, octanoic, and nonanoic acids gave the greatest attractive ranges, each of 1000-fold, over the test dose range examined. All but heptanoic, octanoic, and nonanoic acids were repulsive or elicited no significant response at the highest dose for *O. mercator* (Table 3). For *O. surinamensis*, all fatty acids in high amounts were repulsive except for acetic, heptanoic, octanoic, and nonanoic acids, which were attractive at the 1000-μg dose.

Of four olefinic oat volatiles tested (Table 4), 2(*E*),4(*E*)-heptadienal, 3(*E*),5(*E*)-octadien-2-ol, and 3(*E*),5(*E*)-octadien-2-one had attractive ranges of 0.1–10 μg for *O. mercator*. 2(*E*),4(*E*)-Heptadienal and 3(*E*),5(*E*)-octadien-2-ol were attractive at 0.1–10 μg for *O. surinamensis*. Over the dose range examined for both *Oryzaephilus* spp., 2(*E*),4(*E*)-nonadienal was attractive at 1–100 μg. 3(*E*),5(*E*)-Octadien-2-one was attractive only at the 10-μg dose for *O. surinamensis*.

TABLE 3. RESPONSE BY *O. mercator* AND *O. surinamensis* IN TWO-CHOICE, PITFALL BIOASSAY TO FREE FATTY ACIDS

Test species	Fatty acid[a]	$\bar{X}$% response[b]					
		0.01 μg	0.1 μg	1 μg	10 μg	100 μg	1000 μg
O. mercator	Acetic		9.6	17.4*	47.9***	47.9***	−52.1***
	Propionic		−13.2	7.6	46.2***	32.4*	−44.4***
	Isobutyric		−2.1	19.4*	37.5***	56.3***	−22.2**
	Butyric		7.0	7.6	54.2***	52.4***	−36.1***
	2-Methylbutyric		2.1	27.8*	41.0***	40.3**	−6.9
	Isovaleric	4.5	20.7*	24.3**	57.6***	48.6***	−34.7**
	Valeric		6.9	18.7	53.4***	47.9***	−36.1***
	Hexanoic		−8.4	5.6	57.6***	47.9***	9.7
	Heptanoic		4.9	17.4*	41.6***	41.7**	25.7*
	Octanoic		2.1	11.1	36.8***	36.1**	33.3**
	Nonanoic		−0.7	−3.5	37.5**	42.5***	38.2**
O. surinamensis	Acetic		−2.2	23.0**	42.3***	46.5***	42.9***
	Propionic		14.0	25.8*	26.8**	53.1***	−64.1***
	Isobutyric		−0.7	31.9**	63.6***	5.6	−47.0***
	Butyric		10.0	41.0***	68.0***	31.3**	−52.4***
	2-Methylbutyric		12.8	36.1***	69.8***	−56.0***	
	Isovaleric		9.8	29.1**	39.7***	−52.9***	
	Valeric	−13.2	30.0**	34.2***	85.2***	−9.2	−70.4***
	Hexanoic		12.5	42.3***	−8.0	−50.0***	
	Heptanoic		−7.0	16.0	39.0***	29.5***	26.2*
	Octanoic		−19.7	36.1**	48.2***	64.5***	71.3***
	Nonanoic		3.6	26.0**	48.1***	53.1***	66.2***

[a] Ranked in order of increasing boiling point.
[b] Significant response (*t* test) to experimental stimulus indicated by: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. $N = 12$ replicates per treatment, 12 adults per replicate.

TABLE 4. RESPONSE BY *O. mercator* AND *O. surinamensis* IN TWO-CHOICE, PITFALL BIOASSAY TO SOME OLEFINIC OAT VOLATILES

Test species	Experimental stimulus	$\bar{X}$% response[a]				
		0.01 μg	0.1 μg	1 μg	10 μg	100 μg
O. mercator	2(*E*),4(*E*)-Heptadienal	19.5	39.1***	80.3***	82.4***	13.0
	2(*E*),4(*E*)-Nonadienal	−0.7	16.6	48.6***	48.6***	36.6***
	3(*E*),5(*E*)-Octadien-2-ol	18.4	24.6*	54.0***	71.8***	−8.7
	3(*E*),5(*E*)-Octadien-2-one	−7.6	24.8*	42.9***	74.6***	−10.7
O. surinamensis	2(*E*),4(*E*)-Heptadienal	14.0	31.8**	36.0***	44.7***	−34.2**
	2(*E*),4(*E*)-Nonadienal	16.2	10.6	39.8***	57.3***	56.9***
	3(*E*),5(*E*)-Octadien-2-ol	0.7	25.8*	51.1***	36.1***	−15.7
	3(*E*),5(*E*)-Octadien-2-one	19.4	16.1	0.7	30.3**	3.5

[a] Significant response (*t* test) to experimental stimulus indicated by: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. $N = 12$ replicates per treatment, 12 adults per replicate.

DISCUSSION

Biological Implications. The positive responses to host volatiles in this study suggest that some of these compounds might be used by *Oryzaephilus* spp. as host-finding kairomones in nature. Compared to the cucujolide pheromones and 1-octen-3-ol (A.M. Pierce et al., 1985, 1987, 1989), however, the potential kairomones had relatively higher thresholds for positive response.

The aldehydes and free fatty acids listed in Tables 1 and 3 are ubiquitous natural products in cereals (Maga, 1978) and are common products of lipid oxidation. An increase in levels of free fatty acids is often used as an indicator of deteriorating grain stores (Zeleny, 1954), since grain-infesting fungi (Zeleny, 1954) and bacteria (Kaminski et al., 1980) are well-known producers of free fatty acids. Infestation by *O. surinamensis* in wheat plus dockage resulted in high levels of fat acidity and in infestation by *Penicillium* spp. and bacteria (Sinha, 1983). Because *Oryzaephilus* spp. are commonly associated with a wide variety of stored products of high oil content, it is evidently of adaptive advantage to utilize products of lipid oxidation as host-finding kairomones.

2(*E*),4(*E*)-Heptadienal, 2(*E*),4(*E*)-nonadienal, and 3(*E*),5(*E*)-octadien-2-one have been identified in rancid oat groats (Heydanek and McGorrin, 1981b), while 2(*E*),4(*E*)-nonadienal and 3(*E*),5(*E*)-octadien-2-one have been found in rolled oats (Mikolajczak et al., 1984) and dried oat groats (Heydanek and McGorrin, 1981a), respectively. The above three compounds are probable lipid-autooxidation products (Heydanek and McGorrin, 1981b); 3(*E*),5(*E*)-octadien-2-ol is a likely precursor of 3(*E*),5(*E*)-octadien-2-one.

In their study of oat volatile attractants for *O. surinamensis*, Mikolajczak et al. (1984) found that 2(*E*),4(*E*)-nonadienal was attractive to *O. surinamensis* at only two doses, 0.1 and 1 μg, with repulsion at 100 μg. In the same study, heptanal, hexanal, octanal, and benzaldehyde were moderately attractive, and nonanal was not attractive. Our results (Table 1) indicate lower threshold concentrations and greater active dose ranges than found by Mikolajczak et al. (1984), with the exception of the opposite trend for propanal.

At one test dosage for each compound, propanal, hexanal, hexanoic acid, and nonanoic acid were attractive to *O. surinamensis*, while nonanal and propionic acid were not attractive (O'Donnell et al., 1983). The authors noted that it is unwise to make conclusions about the relative attractancy of such compounds without testing them over a range of doses. In a study of volatile attractants for *O. surinamensis* from carobs, hexanoic acid was more attractive than acetic, isobutyric, butyric, or 2-methylbutyric acids over a range of stimulus dilutions (Stubbs et al., 1985), whereas we found little attraction of *O. surinamensis* by hexanoic acid (Table 3). However, the use of a bioassay based on an insect activity detector in the studies by O'Donnell et al. (1983) and Stubbs et al. (1985) makes comparisons with our results difficult.

Short- and medium-chain aldehydes and fatty acids have been implicated as kairomones for other stored-product insects. Octanal and octanoic acid induced aggregation of larval *Trogoderma glabrum* (Herbst) (Nara et al., 1981). Adults of *Tribolium castaneum* (Herbst) and *Trogoderma granarium* Everts were repelled by C_5–C_9 and C_5–C_8 fatty acids, respectively; valeric acid was a phagostimulant for larvae of *T. granarium* and *Dermestes maculatus* De Geer, while octanoic acid and nonanoic acid were feeding deterrents (Cohen et al., 1974). In comparison with our experiments, these assays were conducted over a very narrow dose range, and further experimentation could well alter the above conclusions.

Practical Implications. The data in Table 2 suggest that, for *O. mercator*, adding nonanal or a mixture of hexanal, octanal, and nonanal to field traps containing cucujolides might be an economical way of lowering the response threshold for the less volatile cucujolides. Adding the highly volatile (*R*,*S*)-1-octen-3-ol to cucujolide mixtures also enhanced the response to the cucujolides in laboratory bioassays for *O. mercator* and *O. surinamensis* (Pierce et al., 1989) and in field traps for *O. surinamensis* (Pierce et al., 1990). Hexanal, octanal, and nonanal in mixture did not interfere with response by *O. mercator* to the individual aldehydes since the response to the three-part aldehyde mixture extended over a greater range, 0.01–100 μg, than response to any of the three individual aldehydes (Tables 1 and 2). Thus, in the absence of pheromones, the three-part aldehyde mixture would be a better trap bait for *O. mercator* than the individual aldehydes.

Other attractive compounds in this study might be more effective as field baits when presented as mixtures. The more volatile, shorter-chain aldehydes such as 2-methylpropanal and pentanal might function over longer distances as useful attractants, while the less volatile, longer-chain compounds such as heptanoic, octanoic, and nonanoic acids might be effective at short range. Because of the limited attractive ranges of some of the potential kairomones, however, release rates would have to be carefully controlled.

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APPLE ERMINE MOTH, *Yponomeuta malinellus* ZELLER[1] Two Components of Female Sex Pheromone Gland Highly Effective in Field Trapping Tests

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Abstract—When electroantennographic responses of male *Yponomeuta malinellus* Zeller to model compounds were determined at dosages of 0.3–30 ng, the strongest responses were obtained from (*Z*)-9-dodecen-1-ol acetate (*Z*9–12 : Ac). Also, strong responses were obtained from (*Z*)-11-tetradecenal (*Z*11–14 : Al) and (*Z*)-11-tetradecen-1-ol (*Z*11–14 : OH). At a dosage of 0.3 ng, *Z*11–14 : Al produced a stronger response than *Z*11–14 : OH, while at a dosage of 30 ng, *Z*11–14 : OH and *Z*11–14 : Al produced equal responses. Gas chromatographic and mass spectral analysis of extracts of female sex pheromone glands showed the presence of *Z*9–12 : Ac, tetradecan-1-ol (14 : OH), (*E*)-11-tetradecen-1-ol (*E*11–14 : OH), *Z*11–14 : OH, hexadecan-1-ol, and hexadecan-1-ol acetate in a ratio of 0.6 : 200 : 37 : 100 : 140 : 35. In field tests, *Z*9–12 : Ac and *Z*11–14 : OH together were required for trap catch, and addition of *Z*11–14 : Al, *E*11–14 : OH, 14 : OH, or (*Z*)-11-tetradecen-1-ol acetate did not increase catch. Ratios in rubber septa of 0.5 : 99.5 to 1.5 : 98.5 (*Z*9–12 : Ac/*Z*11–14 : OH) captured the most males and captures were statistically equivalent for dosages of 10–1000 μg/rubber septum. Traps baited with the synthetic lure produced better catches than those baited with females.

Key Words—Lepidoptera, Yponomeutidae, *Yponomeuta malinellus*, sex pheromone, apple ermine moth, (*Z*)-9-dodecen-1-ol acetate, (*Z*)-11-tetradecen-1-ol.

[1] Lepidoptera: Yponomeutidae.

INTRODUCTION

The apple ermine moth, *Yponomeuta malinellus* Zeller, is a member of a European group of small ermine moths (*Yponomeuta*) that have been studied in recent years in order to clarify taxonomic and evolutionary relationships among species within this genus. This group consists of nine species, of which five are classified as members of the "*padellus* complex" (Wiebes, 1975). The *padellus* complex consists of *Y. padellus*, *Y. cagnagellus*, *Y. mahalebellus*, *Y. malinellus*, and *Y. rorellus*. The other four species are *Y. evonymellus*, *Y. irrorellus*, *Y. plumbellus*, and *Y. vigintipunctatus*. Species of the *padellus* complex are difficult to distinguish morphologically and other features such as feeding preference and sex pheromones may be used for positive identification (Van der Pers and Den Otter, 1978). In flight-tunnel and field tests, attractants have been established for seven of the nine species. Compounds that are involved in the sex pheromones of these species include (*Z*)- and (*E*)-11-tetradecen-1-ol acetates (*Z*11- and *E*11–14 : Ac) and their corresponding alcohols (*Z*11- and *E*11–14 : OH), tetradecyl acetate (14 : Ac) and corresponding alcohol (14 : OH), (*Z*)-11-hexadecen-1-ol acetate (*Z*11–16 : Ac), and hexadecyl acetate (16 : Ac) (Löfstedt and Herrebout, 1988; Löfstedt et al., 1986; Löfstedt and Van der Pers, 1985). Attractants have not been established for *Y. mahalebellus* and *Y. malinellus*.

Although the actual sex pheromone has not been reported for *Y. malinellus*, some studies of its sex pheromone system have been carried out. In flight-tunnel tests, Hendrikse (1986) found that *Y. malinellus* males flew upwind to *Y. evonymellus* females, but terminated response when about 20 cm from them. In measurements of electrophysiological responses to model compounds of single sensilla trichodea of male *Y. malinellus*, van der Pers and den Otter (1978) reported the strongest response was obtained from *Z*11–14 : OH and moderate responses were obtained from *Z*11–14 : Ac, *E*11–14 : Ac, and *Z*9–14 : Ac. In an electroantennographic (EAG) profile of all the normal, monoene, 14-carbon acetates, Hendrikse et al. (1982) reported that *Z*11–14 : Ac gave the strongest response. Although a response profile for the corresponding 14-carbon alcohols or other series was not reported, they reported that *Z*11–14 : OH produced a stronger response than *Z*11–14 : Ac. In single-cell analysis, they found the strongest response from *Z*11–14 : OH and obtained moderate responses from *Z*9,*E*12–14 : Ac, *Z*11–14 : Ac, *Z*11–14 : Al, *Z*11–13 : Ac, *Z*9–13 : Ac and *Z*9–12 : Ac. C. Löfstedt (personal communication) has found *Z*11-, *E*11–14 : OH, and 14 : OH in extracts of female sex pheromone glands, but these compounds did not produce trap catch in field tests. In field tests, *Y. malinellus* have been reported to be captured in traps baited with *Z*11–14 : Ac (Arn et al., 1974; Stockel, 1981).

Y. malinellus occur in both Europe and Asia. Our interest in *Y. malinellus*

developed because of its detection in nursery stock in British Columbia, Canada, in 1981 and subsequently in Washington State, U.S.A in 1985 (Douglas J. Parker, personal communication). Thus, an effective attractant was needed to detect and monitor populations of *Y. malinellus* as part of a program attempting to prevent its spread into commercial apple orchards. Here we report the development of a highly effective lure based on a mixture of *Z*11–14 : OH and *Z*9–12 : Ac.

METHODS AND MATERIALS

Insects. Larvae and pupae of *Y. malinellus* in their tents were collected from an infested apple tree near Bellingham, Washington, U.S.A., placed in sealed containers, and transported to a quarantine rearing facility in Yakima. The tents were divided into parts, each containing several cocoons, and each part was placed on moist peat moss in a closed container. The room was maintained at 50% relative humidity and 20°C with a 16 : 8 light–dark cycle. Adults were collected daily 3 hr after onset of photophase and sexed. The females were maintained in separate glass vials while the males were maintained together in cages, and both sexes were provided with a 10% sugar solution.

Collection of Pheromone. Calling female moths (6–7 days old) were collected 3–4 hr into photophase and chilled in a refrigerator for about 10 min to inactivate them. Terminal abdominal segments were severed and steeped for 15 min in dichloromethane, and the solution was removed with a syringe and stored at −20°C until used.

EAG. The experimental apparatus was the same as previously described (McDonough et al., 1980). Test cartridges for the EAG profiles were loaded with 60 μg of model compound. Duplicate determinations were made for each datum point both in the EAG profile and in the concentration study. The model compounds were available commercially or were prepared and purified by established synthetic methodology (Voerman, 1988). They were at least 98% pure by gas chromatographic analysis, and contained 1% or less of the geometrical isomer.

GC-EAG. Fractions of female sex pheromone gland (SPG) extract for EAG analysis were collected from a GC equipped with a megabore capillary column (methyl silicone; DB-1; 15 m × 0.53 mm ID; J & W Scientific, Folsom, California) connected to a glass-lined splitter sending one part of effluent to the detector and nine parts to the collector. The GC was programmed at 2°/min from 120 to 210°C. Ten fractions encompassing the retention time of 10 : OH through 18 : Ac were collected as follows, (fraction) time interval in min: (1) 5–15.5; (2) 15.5–18; (3) 18–21; (4) 21–24; (5) 24–27.5; (6) 27.5–32; (7) 32–37.5; (8) 37.5–43; (9) 43–47; (10) 47–53. The retention times in minutes, of synthetic reference compounds were: 10 : OH, 5.5; 12 : OH, 7.9; 12 : Ac, 17.3;

Z11–14:OH, 21.8; 14:Ac, 31.0; 16:Ac, 42.2; 18:OH, 47.1; 18:Ac, 51.7. GC analysis of SPG extract was accomplished with a capillary column (methyl silicone; DB-1; 60 m × 0.25 mm; J & W Scientific) operated at 180°C.

LC. An extract of 150 female SPG was chromatographed on a column of silica gel (0.6 g). The column was eluted successively with hexane, hexane–dichloromethane (70:30), and dichloromethane. The hexane–dichloromethane (70:30) and dichloromethane fractions containing esters and alcohols were combined and concentrated for GC-MS analysis.

GC-MS. A Hewlett-Packard (Avondale, Pennsylvania) gas chromatograph (model 5790) with a quadruple mass selective detector (model 5970) was equipped with a capillary column (DB-Wax; 60 m × 0.25 mm ID; J & W Scientific). When the gland extract of 10 female *Y. malinellus* was analyzed, the column was held at 80°C for 2 min and programmed at 20°/min to 200°C and maintained at that temperature. Total ion abundance from $m/z = 15$ to $m/z = 300$ was monitored. When the cleaned-up extract of 150 females was analyzed in the single ion monitor (SIM) mode, the same temperature program was used, but ions 61 and 166 were monitored between 12 and 17 min (Z9–12:Ac at 12.78 min); ions 68, 166, and 194 were monitored between 17 and 20 min (Z11–14:OH at 18.10 min); and ions 81 and 210 were monitored between 20 and 28 min (*E*10,*E*12–14:OH at 24.03 min).

Field Tests. Field tests were conducted in a block of 18 apple trees near Bellingham, Washington. Test chemicals were dissolved in rubber septa (West Co., Phoenixville, Pennsylvania), and placed in Pherocon 1C traps (Trece, Corp., Salinas, California). The traps were hung in the upper one-third of the canopy of each tree. Three replicates of six test lures were randomly drawn for trap positions. Each week the number of males in the traps was counted and the traps were replaced. In the test with female *Y. malinellus* as the bait, three females in a screened cage were placed in each trap.

RESULTS

EAG. EAG response profiles of male *Y. malinellus* to the *n*-alkyl and *n*-alkenyl 12- and 14-carbon alcohols and acetates are given in Figure 1. The values are expressed as a percent of the response to Z9–12:Ac, which produced the strongest response. Z11–14:OH was comparable to, but not as strong as, Z9–12:Ac (87%). Z11–14:Ac produced a stronger response than other 14-carbon acetates (76%) but was weaker than Z11–14:OH. The EAG profile for the 14-carbon acetates has been reported previously (Hendrikse et al., 1982), and our results are in agreement with theirs. A few other compounds were also available for testing. These were the (Z)- and (*E*)-7-, 9-, and 11-tetradecenals and hexadecenals and the (Z)- and (*E*)-7-, 9-, and 11-hexadecenyl alcohols and

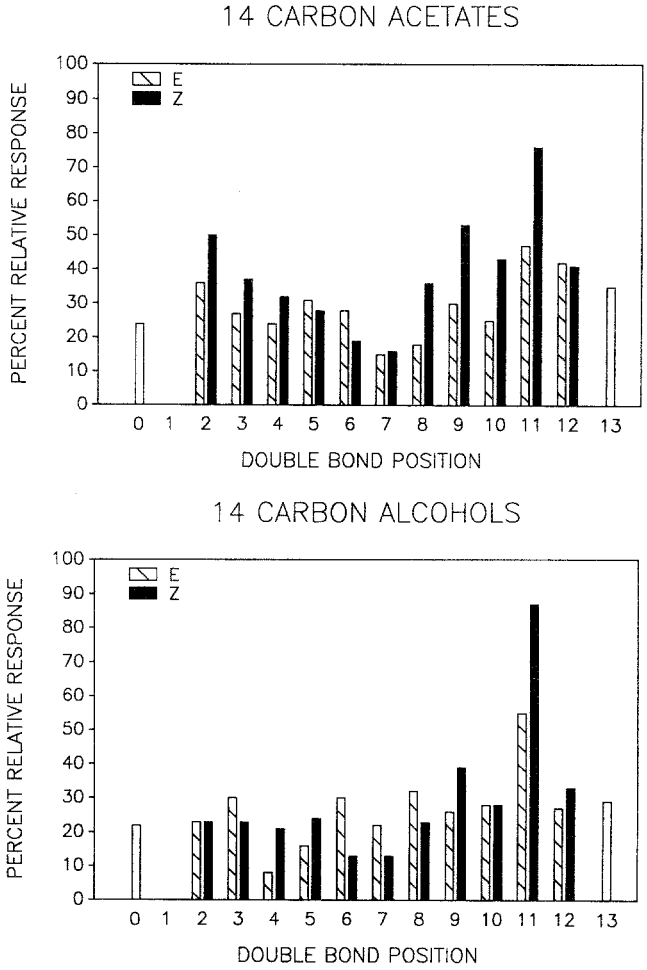

FIG. 1. Electroantennographic responses of male apple ermine moth (*Yponomeuta malinellus*) to model compounds. Numbers indicate double-bond position; zero indicates saturated.

acetates. Of this group, only Z11–14 : Al produced a strong response (85% of Z9–12 : Ac).

To better evaluate the relative potency of the compounds producing strong EAG responses, EAG measurements were made at dosages of 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 ng/test cartridge. Z11–14 : Al, Z11–14 : OH, and Z9–12 : Ac gave excellent linear correlations for EAG response versus logarithm of the dosage (Table 1). The intercepts (y_0), which are the responses in mV at a dos-

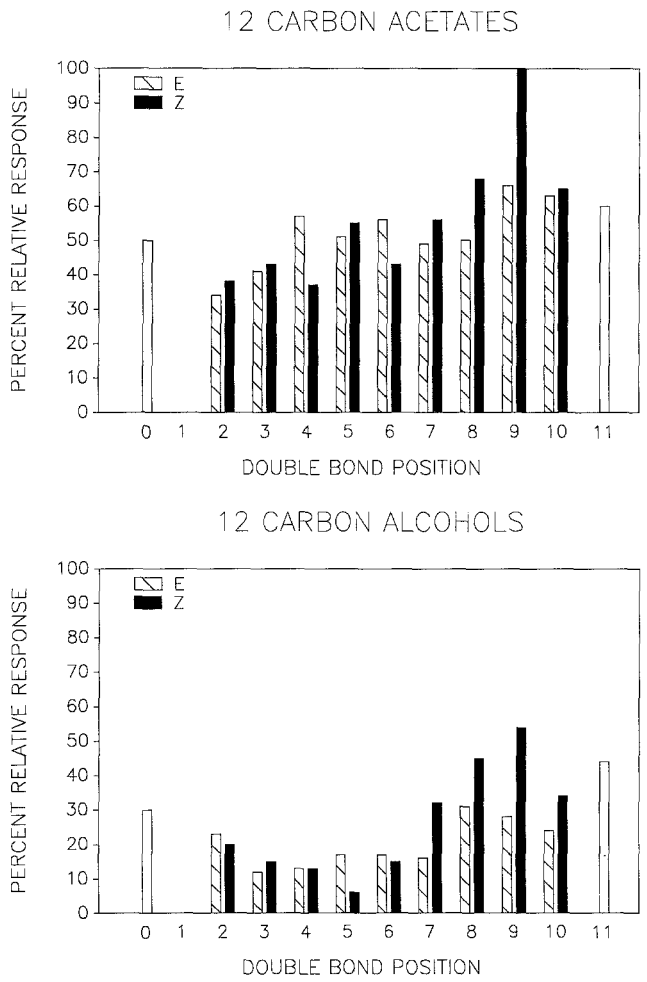

FIG. 1. (*Continued*)

age of 1 ng, showed the order to be Z9-12:Ac > Z11-14:Al > Z11-14:OH > Z11-14:Ac. Because of the differing slopes (*m*), Z11-14:OH and Z11-14:Al produced approximately equivalent responses at dosages above 30 ng.

GC-EAG and GC-MS. Gas chromatographic fractions of extract of sex pheromone glands of 59 female *Y. malinellus* covering the volatility range from the retention time of 10:OH through 18:Ac were collected from a methyl silicone column, and the EAG response to each fraction was determined. Fraction 4, containing the 14-carbon alcohols, produced the strongest response (0.8 mV above the blank). A capillary GC trace (methyl silicone column) of extract indicated the presence of 14:OH, *E*11-14:OH, and Z11-14:OH in this frac-

TABLE 1. LINEAR REGRESSION PARAMETERS FOR EQUATION $E = m \ln d + y_0$[a]

Compound	y_0	m	r^2
Z11-14:OH	0.442	0.200	0.96
Z11-14:Al	0.620	0.152	0.996
Z9-12:Ac	0.960	0.234	0.992
Z11-14:Ac	0.287	0.074	0.82

[a] E is the electroantennographic response in millivolts, m is the slope of the line, ln d is the natural logarithm of the dosage (over the range 0.3–30 ng) and y_0 is the intercept.

tion. The other fractions produced responses ranging from 0.2 to 0.4 mV. Fraction 2, which would contain Z9-12:Ac and Z11-14:Al if present, produced the 0.2-mV response.

A GC-MS analysis of the extract of SPGs from 10 females showed the presence of the following compounds in the given ratios: 14:OH (2.0), E11-14:OH (0.37), Z11-14:OH (1.0), 16:OH (1.4), and 16:Ac (0.35). The amount of Z11-14:OH in the extract was 5.8 ng. An extract of SPGs from 150 females was partially purified by liquid chromatography. The alcohol and ester fractions were combined, concentrated, and analyzed by GC-MS (SIM). The ions monitored were chosen so as to be able to detect Z9-12:Ac, Z11- and E11-14:OH, and E10,E12-14:OH. The analysis indicated 14.9 ng of Z11-14:OH, 6.5 ng of E11-14:OH, 0.09 ng Z9-12:Ac (0.6% of Z11-14:OH; retention time 12.794 min; ratio of 61/166 ions = 43:57; retention time of Z9-12:Ac standard was 12.795 min; ratio of 61/166 ions = 45:55; limit of detection 0.05 ng), and no E10,E12-14:OH.

Field Tests. In test 1 (Table 2), traps baited with Z11-14:OH + E11-14:OH, did not catch significant numbers of males, whereas traps baited with the three-component lure, Z11-14:OH + E11-14:OH + Z9-12:Ac caught large numbers of males. When the dosage was 100:30:1, catches exceeded those of traps baited with females. All of the females were alive at the end of the test. Traps baited with the three-component lure caught as many males as the traps baited with the three components plus Z11-14:Al (test 2). Test 3 showed that both Z11-14:OH and Z9-12:Ac must be present to produce trap catch, but the presence or absence of E11-14:OH had no effect on trap catch. Various combinations of Z11-14:OH + E11-14:OH + Z11-14:Ac did not produce trap catch even though Z11-14:OH + E11-14:OH + Z9-12:Ac produced a very high capture (test 4). Dosages of Z11-14:OH + Z9-12:Ac (100:1), ranging over a factor of 100, produced equivalent catch (test 5). Also, ratios of Z11-14:OH to Z9-12:Ac of 99.5:0.5 to 98.5:1.5 and perhaps to 97.5:2.5 produced equivalent trap catch (test 6), and ratios of 99.7:0.3 and 97:3 produced lower catch (test 1).

TABLE 2. CAPTURES OF ADULT MALE *Y. malinellus* IN TRAPS BAITED WITH VARIOUS CANDIDATE LURES[a]

Test No.	Lure	Dosage (μg/septum)	Cumulative captures
1	July 13–19, 1988		
	Z11-14:OH + E11-14:OH	100:30	1c
	Z11-14:OH + E11-14:OH + Z9-12:Ac	100:30:0.3	53b
	Z11-14:OH + E11-14:OH + Z9-12:Ac	100:30:1.0	163a
	Z11-14:OH + E11-14:OH + Z9-12:Ac	100:30:3.0	84b
	Females		88b
	Blanks		0c
2	July 20–26, 1988		
	Z11-14:OH + E11-14:OH + Z9-12:Ac	100:30:1	92a
	Z11-14:OH + E11-14:OH + Z9-12:Ac + Z11-14:Al	100:30:1:0.1	56a
	Z11-14:OH + E11-14:OH + Z9-12:Ac + Z11-14:Al	100:30:1:0.3	94a
	Z11-14:OH + E11-14:OH + Z9-12:Ac + Z11-14:Al	100:30:1:1.0	90a
	Z11-14:OH + E11-14:OH + Z9-12:Ac + Z11-14:Al	100:30:1:3.0	117a
	Blanks		0b
3	July 27–August 1, 1988		
	Z11-14:OH + E11-14:OH + Z9-12:Ac	100:30:1	70a
	Z11-14:OH + Z9-12:Ac	100:1	79a
	E11-14:OH + Z9-12:Ac	30:1	0b
	Z9-12:Ac	1	0b
	Z9-12:Ac	10	0b
	Blanks		0b
4	August 1–7, 1988		
	Z11-14:OH + E11-14:OH + Z9-12:Ac	100:30:1	264a
	Z11-14:OH + E11-14:OH + Z11-14:Ac	100:30:0.75	1b
	Z11-14:OH + E11-14:OH + Z11-14:Ac	100:30:2.25	0b
	Z11-14:OH + E11-14:OH + Z11-14:Ac	100:30:7.5	0b
	Z11-14:OH + E11-14:OH + Z11-14:Ac	100:30:22.5	0b
	Blanks		0b
5	August 8–14, 1988		
	Z11-14:OH + Z9-12:Ac	10:0.1	74a
	Z11-14:OH + Z9-12:Ac	30:0.3	78a
	Z11-14:OH + Z9-12:Ac	100:1	81a
	Z11-14:OH + Z9-12:Ac	300:3	108a
	Z11-14:OH + Z9-12:Ac	1,000:10	92a
	Blanks		0b
6	August 15–22, 1988		
	Z11-14:OH + Z9-12:Ac	300:1.5	17a
	Z11-14:OH + Z9-12:Ac	300:3.0	19a
	Z11-14:OH + Z9-12:Ac	300:4.5	21a
	Z11-14:OH + Z9-12:Ac	300:6.0	12ab
	Z11-14:OH + Z9-12:Ac	300:7.5	7ab
	Blanks		1b

[a] There were three replicates per test lure. In each test numbers followed by the same letter were not significantly different ($P = 0.05$) by Duncan's multiple-range test.

DISCUSSION

The EAG studies of model compounds, GC-EAG, GC-MS, and field tests, indicate *Z*11–14 : OH and *Z*9–12 : Ac are sex pheromone components of *Y. malinellus*. The failure of the GC-EAG experiment to show a strong response to the fraction containing *Z*9–12 : Ac is not inconsistent with this conclusion. Based on the 0.8-mV response of the GC-EAG fraction containing *Z*11–14 : OH, the percentage of *Z*9–12 : Ac relative to *Z*11–14 : OH in the GC-MIS (SIM) experiment, and the parameters of Table 1, a value of 0.2 mV, the same as was found, would be predicted for the GC-EAG fraction containing *Z*9–12 : Ac. Although *E*11–14 : OH was present in SPG extracts, it had no effect on trap catch. Thus, its possible significance in the communication system is not clear. Because *E*11–14 : OH could be a biosynthetic precursor to *E*10,*E*12–14 : OH (Roelofs and Brown, 1982; Löfstedt and Bengtsson, 1988), the GC-MS (SIM) experiment was designed to detect its possible presence, but with negative results.

It is not known whether the North American *Y. malinellus* was introduced from Europe or Asia. It is also not known whether populations from these two areas differ, since studies of the sex pheromone system of *Y. malinellus* from Asia have not been reported. In Europe *Y. malinellus* is very closely related to *Y. padellus* (Nei's genetic distance is 0.083), and therefore these species are considered to have undergone evolutionary divergence from a *padellus*-like ancestor rather recently (Arduino et al., 1983; Arduino and Bullini, 1985). From their study of the sex pheromones of several *Yponomeuta* species, Löfstedt and Van der Pers (1985) suggested that *Z*11- and *E*11–14 : Ac + *Z*11–14 : OH comprised the sex pheromone of the ancestor to the present *Yponomeuta*. The structure of the sex pheromone of the American *Y. malinellus* appears to be consistent with these proposals. Slight redirections in the actions of the Δ11 desaturase and chain-shortening enzymes (Roelofs and Brown, 1982) of the *padellus*-like ancestor would be sufficient to develop the new pheromone systems of *Y. padellus* and *Y. malinellus*.

Lure longevity was not tested. However, it is possible to estimate the field life of the lure based on half-lives and effect of temperature on half-lives (Butler and McDonough, 1979, 1981; McDonough et al., 1989). In Bellingham, Washington, the average temperature during the 1988 summer was 17.5°C (Bellingham Public Works Department, private communication). Taking $t_{1/2}$ = 33 day at 20°C for *Z*9–12 : Ac and $t_{1/2}$ = 117 days at 20° for *Z*11–14 : OH and assuming lure longevity is limited by the time for the *Z*11–14 : OH/*Z*9–12 : Ac ratio to change from 98.5 : 1.5 to 99.5 : 0.5, we calculated longevities of 73 days at 20°C. Thus, a lure consisting of 200 μg *Z*11–14 : OH + 3 μg *Z*9–12 : Ac should provide a field life of 10 weeks without loss of efficiency.

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SUCROSE ESTERS OF CARBOXYLIC ACIDS IN GLANDULAR TRICHOMES OF *Solanum berthaultii* DETER SETTLING AND PROBING BY GREEN PEACH APHID

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Abstract—Removal of type B trichome exudate from *Solanum berthaultii* leaflets leads to a decrease in tarsal gumming and mortality and an increase in feeding by the green peach aphid, *Myzus persicae*. Type B trichome exudate of the *S. berthaultii* accession PI 473331 is composed of a complex of 3′,3,4,6-tetra-*O*-acyl sucroses containing primarily short-chain branched carboxylic acids. The acyl constituents are primarily derived from 2-methylpropanoic, 2-methylbutyric, and 8-methylnonanoic acids but constituents derived from *n*-decanoic and dodecanoic acids are also present. Sucrose esters inhibit settling and probing by aphids in glass feeding cages.

Key Words—*Solanum berthaultii*, *Myzus persicae*, green peach aphid, Homoptera, Aphididae, sucrose esters of carboxylic acids, glandular trichome exudate, plant resistance to insects.

INTRODUCTION

The wild potato, *Solanum berthaultii* Hawkes, is defended against a variety of arthropod pests by the presence of two types of glandular trichomes on its foliage (Dimock and Tingey 1985; Gibson 1971, 1974; Tingey and Laubengayer, 1981, 1986; Tingey and Sinden, 1982). Type A trichomes are 120–210 μm in length with 50- to 70-μm diameter tetralobulate heads (Gregory et al., 1986).

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Upon rupture, these trichomes release an exudate that rapidly polymerizes (Ryan et al., 1982) and adheres to the tarsi and mouthparts of insects (Gibson and Turner, 1977; Tingey and Gibson, 1978). Small insects such as aphids may become entrapped on the leaf surface. The longer type B trichomes are 600–950 μm in length and secrete a viscous fluid that forms a droplet up to 45 μm in diameter on the tip of each stalk (Tingey, 1985). Type B exudate is important in the resistance of *S. berthaultii* to the green peach aphid, *Myzus persicae* (Sulzer). Transfer of type B exudate to foliage of the susceptible cultivated potato, *Solanum tuberosum* L., results in fewer probes, increased time to first probe, and reduced total feeding activity by *M. persicae* (Lapointe and Tingey, 1984). Type B exudate also has an interactive effect with type A trichomes. Aphids are more likely to rupture type A trichomes if their tarsi are coated with viscous type B exudate (Tingey and Laubengayer, 1981).

Trichomes of other plants of the family Solanaceae also produce viscous exudates that affect aphid behavior. Exudate from type IV trichomes of *Lycopersicon pennellii* Corr. (D'Arcy), a wild relative of the cultivated tomato, deters settling by the potato aphid, *Macrosiphum euphorbiae* (Thomas) (Goffreda et al., 1988). The active components of this exudate are glucose esters of branched chain carboxylic acids (Burke et al., 1987; Goffreda et al., 1988, 1989). Sucrose esters of carboxylic acids present in glandular trichomes of tobacco, *Nicotiana tabacum* L. are toxic to larvae of the tobacco budworm, *Heliothis virescens* (F.) (Cutler et al., 1986).

Recently, several tri- and tetra-*O*-acylated sucroses have been identified in exudate of *S. berthaultii* type B glandular trichomes (King et al., 1986; 1987a,b). We report here the performance of *Myzus persicae* on *S. berthaultii* after removing type B trichome exudate; the collection, isolation, and identification of carboxylic acid sucrose esters from type B exudate of *S. berthaultii* accession PI 473331; and describe the influence of sucrose esters on the behavioral activity of the green peach aphid.

METHODS AND MATERIALS

Identification and Isolation of Sucrose Esters. Seed of *Solanum berthaultii* (PI 473331) was obtained from the Interregional Potato Introduction Station (Sturgeon Bay, Wisconsin) and germinated in the greenhouse using peat–vermiculite media. Seedlings were transplanted in late May to field plots of Rhinebeck slit loam at the H.C. Thompson Vegetable Research Farm, Freeville, New York. Two bands of NPK fertilizer (168-168-168 kg [AI]/ha) were applied to each row before planting. EPTC (Eptam 10G) granules were applied at 67 kg/ha in July for weed control. Chlorothalonil (Bravo 500) was applied at 2.3

liters/ha with a hydraulic boom sprayer at 10-day intervals throughout the growing season for control of late blight.

Exudate of type B trichomes was collected in August by briefly dipping excised foliage in methanol. The leaf rinse was filtered, reduced to dryness *in vacuo* at 35°C, and partitioned between 25% aqueous methanol and hexanes. The aqueous phase was taken to dryness, dissolved in a minimal volume of chloroform, and fractionated by flash column chromatography (Still et al., 1978) on a 3 × 15-cm silica gel column eluted with a gradient from chloroform to 30% methanol. The fraction containing sucrose esters was fractionated further by a second passage over a silica gel column 1.5 × 15 cm, eluted with a gradient from chloroform to 10% methanol. Individual sucrose esters were purified by reverse-phase HPLC (Waters 5μ C18 radial compression; 17% aqueous acetonitrile, at 2 ml/min; detection at 210 nm).

Carboxylic acid compositions were determined from sucrose esters transesterified in ethanol using a J&W Scientific (Folsom, California) 30-m DB-FFAP capillary column. [^{1}H]- and [^{13}C] NMR experiments were performed on a Varian XL-400 instrument in $CDCl_3$. COSY spectra were recorded using double precision acquisition with a 4000-Hz spectral width, 128 τ_1 increments, and the spectra were zero-filled to a 512 × 512 data point set. Recycle time was 2 sec between each four transients per τ_1 increment. Fast atom bombardment collisionally activated dissociation (CAD) positive ion mass spectra were acquired on a Finnigan triple quadrupole mass spectrometer. The sample was bombarded with a beam of 6–8 kV cesium ions generated from a cesium ion gun. High-resolution fast atom bombardment spectra were acquired on a VG Instruments 70-250 SEQ in positive ion mode.

Aphid Rearing. A colony of *Myzus persicae* was initiated from a single nymph collected in July 1987 from field plots of the cv. Katahdin at Freeville, New York. The colony was maintained in a controlled environment chamber at 25°C, 16:8/light-dark photoperiod (250 μE/mm^2/sec) on excised terminal mainstems of *S. tuberosum* cv. Katahdin.

Removal of Type B Exudate Droplets. A number of solvents were tested for ability to rapidly and quantitatively remove the exudate droplets from type B trichomes while minimally disrupting the leaflet. Halogenated solvents are effective in removing trichome exudate but also remove cuticular components (King et al., 1987a,b) and cause rapid wilting and death of the leaflet. Type B droplets extract poorly in water (Holley et al., 1987). Alcohols (methanol or ethanol) are effective in type B droplet removal and cause minimal disruption of the leaf; type B trichome droplets begin to regenerate within 48 hr after removal with ethanol. Ethanol treatment does not affect polyphenol oxidase activity (Avé et al., 1986) of the type A trichome, while methanol causes a 10–15% decrease in oxidative activity (Neal and Tingey, unpublished data).

Biological Activity of Type B Droplets. Field grown plants (*S. berthaultii* accession PI 473331) were propagated by nodal cuttings and grown in a greenhouse under high intensity illumination (3600 E/mm^{2}sec) and 16:8/light–dark photoperiod. *S. tuberosum* cv. Katahdin plants (which lack type B droplets) were grown from tubers. Pairs of opposite leaflets on 10 leaves from three *S. berthaultii* plants were selected. For each pair, one leaflet was untreated, while type B droplets were removed from the other by dipping in ethanol for about 5 sec. As a control, the effects of the ethanol dip were measured using paired leaflets (one of each pair was dipped) on five leaves of a cv. Katahdin plant. Aphid nymphs were caged (2/cage) in 16-mm-diameter glass ring cages and held for 73 hr. The position of the aphids relative to the leaflet, whether or not they were in a feeding position, and mortality were recorded hourly for the first 8 hr and two to three times daily on days 2 and 3. Mortality and fraction feeding were compared by *t* test with single cages as replicates. After 73 hr, all aphids were evaluated for the presence of type A trichome exudate on the tarsi, and the diameter of the exudate accumulation measured at 30× magnification using an optical micrometer. The numbers of tarsi with exudate for aphids on dipped and untreated leaflets were compared by *t* test. The diameters of the exudate (for coated tarsi only) were compared for aphids on treated and untreated leaflets and for dead aphids and live aphids irrespective of treatment by *t* test.

Biological Activity of Sucrose Esters. Fourth instars and apterae were confined in groups of five using glass ring cages 25 mm diameter × 20 mm high. A Parafilm membrane stretched over a second ring cage served as the top of the chamber. Aphids were placed in each cage and allowed to probe inverted through the membrane for feeding on a solution of 20% sucrose. Ten cages were prepared for each treatment.

Behavioral activity of sucrose esters was determined by applying acetone solutions of the sucrose ester mixture to the membrane surface adjacent to the aphid. Control areas were treated with acetone alone (4 μl/cm^2). The acetone solution was spread as it dried to produce a uniform surface coating. For choice tests, a contiguous half of the surface was treated. Six concentrations of the sucrose ester mixture (100, 30, 10, 3, and 1 μg/cm^2) were tested. This is within the range present on the leaf; 10–20 μg/cm^2 were recovered from the leaf surface.

A second series of experiments was designed to determine the site of action of the sucrose esters on the green peach aphid. In these studies, the membrane was treated on the side opposite the aphid so that contact would be limited to the stylets. A choice test (concentration of 100 μg/cm^2) was compared to a sham-divided acetone-treated control, and a no-choice treatment (100 μg/cm^2) was compared to acetone alone and sucrose esters (100 μg/cm^2) on the side of the membrane adjacent to the aphid.

In both experiments, two parameters, i.e., settling and number of stylet sheaths, were measured. Settling was scored as the mean fraction of aphids resting on a membrane surface at each observation period. At least four observations were made at intervals not less than 2 hr apart during each 48-hr experiment. After the last observation, the sucrose diet solution was decanted and replaced with a solution of safranin O in methanol to stain stylet sheaths red. The parafilm membranes were inverted onto a grid, excess safranin removed with water, and the number of sheaths in a 50-mm^2 area counted at 25× magnification.

RESULTS

Effect of Type B Droplet Removal on Myzus persicae. Ethanol dip of *S. tuberosum* leaflets had no effect on feeding or mortality of aphids. Overall 72-hr mortality on *S. tuberosum* was 5% ($N = 19$). Removal of type B droplets from *S. berthaultii* significantly increased the fraction of aphids feeding for 36 hr after treatment (Figure 1). At 48 hr, type B trichome droplets began to regenerate on the dipped leaflets, and the fraction of aphids feeding declined. The 72-hr mortality on *S. berthaultii* was significantly higher ($P = 0.003$) on the untreated leaflets (67%) than on the dipped leaflets (20%). The average number of tarsi encased with type A trichome exudate per aphid was significantly higher ($P = 0.024$) for aphids on untreated leaflets (2.8) than aphids on dipped leaflets (1.4). There was no difference in the average diameter of the exudate residue ($P = 0.17$) for aphids on dipped and untreated leaflets. However, the size of the exudate accumulation was greater ($P = 0.0002$) on dead aphids (57 μm/coated tarsus) than on live aphids (35 μm/coated tarsus). All dead aphids were found on the cage and had left the leaf surface before expiring.

Identification of Sucrose Esters in Type B Trichome Exudate. HPLC analyses demonstrate that sucrose esters are the predominant components in type B trichome exudate of *S. berthaultii.* The primary acyl constituents were 2-methylpropanoic (45%), 2-methylbutyric (28%), 8-methylnonanoic (23%), *n*-decanoic (2%), and dodecanoic acids (2%) and were identified as their ethyl esters. Although the ester complex is heterogeneous by HPLC, [^{1}H]-NMR spectra of the region δ 5.5–3.0 of both the sucrose ester complex and purified sucrose esters were identical, showing that all members of the complex were similarly esterified. COSY spectra were effective in identification of overlapping coupled spin systems. Three positions of esterification were indicated by three downfield shifted resonances besides H-1 of glucose (δ 5.48, d, $J = 3.4$ Hz) in [^{1}H] NMR spectra: δ 5.25, t, $J = 9.6$ Hz (H-3); δ 5.1, t, $J = 9.6$ Hz (H-4); δ 5.15, d, $J = 8.0$ Hz (H-3′). Although cross-peaks from H-5 to H-6 were not detected in

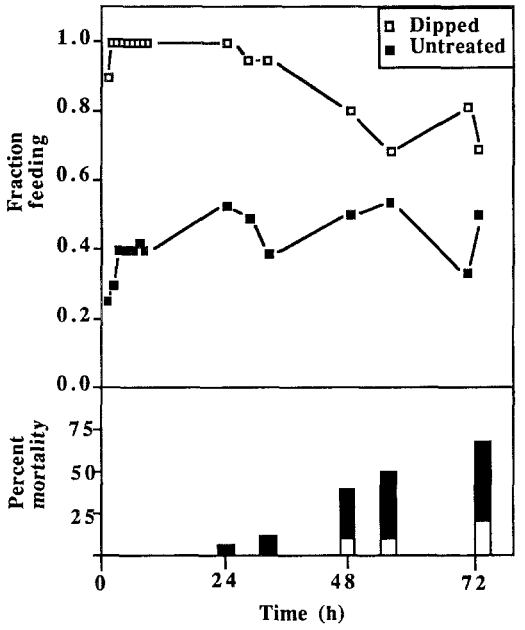

FIG. 1. Mortality and fraction of aphids feeding on *Solanum berthaultii* leaflets from which the type B exudate has been removed. Mortality at all time intervals and fraction feeding at 1 through 32 hr is significantly different for treated versus untreated leaflets (G test of independence, $P = 0.05$). At 48 hr, droplets on treated leaflets began to reappear.

COSY spectra, the singlet at δ 4.20 suggested an additional point of acylation at C-6 (King et al., 1987a). Four carbonyl resonances were apparent in ^{13}C spectra of a purified sucrose ester (δ 176.47, 174.95, 174.56, and 173.44). Other ^{13}C signals for the sucrose skeleton were nearly identical to those reported by King et al. (1987a) for 3',3,4,6-tetra-*O*-acyl sucroses in other *S. berthaultii* accessions.

Fast atom bombardment (fab) mass spectra (positive ions) of the sucrose ester complex gave major high-mass ions (Na^+ adducts) at *m/z* 757, 743, and 729. High-resolution fab of *m/z* 743 confirmed tetra-*O*-acylation (*m/z* 743.3859, $C_{35}H_{60}O_{15}Na^+$, calc. 743.3829); GC analysis indicated the presence of 3-methylpropanoic, 2-methylbutyric, and 8-methylnonanoic acids. CAD (positive ions) of the *m/z* 703 ion ($M + H - H_2O$) in a triple quadripole mass spectrometer gave a fragment of *m/z* 471 representing the triacylated glucopyranosyl cation bearing 3-methylpropanoic, 2-methylbutyric, and 8-methylnonanoic sub-

stituents; losses of the neutral acids were evident as ions *m/z* 383 (13%), 369 (22%), and 299 (13%). CAD of the *m/z* 743 Na^+ adduct further indicated that the fructose moiety was acylated with 3-methylpropanoic acid by the appearance of the *m/z* 273 (87%) 3-methylpropanoic acid fructose ester Na^+ adduct ion. Neutral loss of the 3-methylpropanoic acid fructose ester fragment gave rise to the tri-O-acylglucose Na^+ adduct bearing 2-methyl-propanoic, 2-methylbutyric, and 8-methylnonanoic acids (*m/z* 511, 60%). Other significant ions in the CAD spectrum of *m/z* 743 were *m/z* 655 (10%), 641 (8%), 493 (92%), 423 (12%), 409 (8%), 405 (20%), 391 (19%), and 255 (10%).

Activity of Sucrose Esters against Myzus persicae. In all artificial feeding cage trials, aphid survival over the 48-hr observation period exceeded 95%. At a concentration of 100 $\mu g/cm^2$, sucrose esters on the side adjacent to the test aphids significantly deterred aphid settling and reduced probing in both choice (Table 1) and no choice tests (Table 2). Deterrence declined with decrease in concentration; significant deterrent activity was still present at 33 $\mu g/cm^2$, but not at 10 $\mu g/cm^2$. While a concentration of 30 $\mu g/cm^2$ provided uniform membrane coverage, coverage was splotchy at 10 $\mu g/cm^2$. Sucrose esters coated on the membrane side opposite the aphids did not influence settling or probing in either choice (Table 3) or no choice assays (Table 2). Settling and probing behaviors were affected only when aphids were in physical contact with the sucrose esters.

TABLE 1. DETERRENCE OF APHID SETTLING AND PROBING (STYLET SHEATH PRODUCTION) IN CHOICE TESTS BY UNIFORMLY COATING HALF THE AREA OF DIET CAGE MEMBRANES (ON THE SAME SIDE AS APHIDS) WITH SUCROSE ESTERS FROM TYPE B GLANDULAR TRICHOMES OF *Solanum berthaultii* ACCESSION (PI 473331)

Concentration ($\mu g/cm^2$)	Fraction settled[a]			No. sheaths/mm^2[b]		
	Treated	Untreated	P[c]	Treated	Untreated	P[d]
100	0.12	0.53	0.0003	0.21	2.24	0.0001
33	0.14	0.49	0.0018	0.38	3.36	0.0001
10	0.28	0.45	0.19	1.31	1.77	0.06
3	0.41	0.34	0.58	1.39	1.17	0.33
1	0.34	0.48	0.31	1.66	1.30	0.15

[a] N = 50 aphids (5 aphids/cage, 10 replications).
[b] Based on counts of 50 mm^2 from each of four cages.
[c] Probability that sucrose esters have no effect on the distribution of aphids by G test, goodness of fit.
[d] Probability that sucrose esters have no effect on the distribution of stylet sheaths by G test, goodness of fit.

TABLE 2. EFFECT OF COATING DIET CAGE MEMBRANES ON SIDE ADJACENT TO APHIDS OR OPPOSITE APHIDS WITH 100 μm/cm^2 SUCROSE ESTERS FROM *Solanum berthaultii* ACCESSION (PI 473331) ON DETERRENCE OF APHID SETTLING AND PROBING (STYLET SHEATH PRODUCTION) IN NO-CHOICE ASSAY

Treatment	Fraction settled[a]	No. sheaths/mm^2	(± SE)[b]
Adjacent	0.17a[c]	0.23x[d]	(0.08)
Opposite	0.61b	0.54xy	(0.24)
None	0.56b	1.16y	(0.10)

[a]N = 50 aphids (5 aphids/cage, 10 replications).
[b]Based on counts of 50 mm^2 from each of three cages.
[c]Values followed by the same letter do not have significant differences in fraction of aphids settled by G test of independence (P = 0.95).
[d]Values followed by the same letter are not significantly different by LSD statistic ($P > 0.05$).

TABLE 3. DETERRENCE OF APHID SETTLING AND PROBING (STYLET SHEATH PRODUCTION) IN CHOICE TESTS BY UNIFORMLY COATING HALF THE AREA OF DIET CAGE MEMBRANES (ON THE OPPOSITE SIDE FROM APHIDS) WITH 100 μg/cm^2 SUCROSE ESTERS FROM TYPE B GLANDULAR TRICHOMES OF *Solanum berthaultii* ACCESSION (PI 473331)

	Fraction settled[a]			No. sheaths/mm^2[b]		
	Treated	Untreated	P[c]	Treated	Untreated	P[d]
Treatment	0.28	0.30	0.85	1.1	1.3	0.14
Sham divided	0.30	0.26	0.79			

[a]N = 50 aphids (5 aphids/cage, 10 replications).
[b]Based on counts of 50 mm^2 from each of three cages.
[c]Probability that sucrose esters have no effect on the distribution of aphids by G test, goodness of fit.
[d]Probability that sucrose esters have no effect on the distribution of stylet sheaths by G test, goodness of fit.

DISCUSSION

While the sucrose esters from glandular trichomes of *N. tabacum* are toxic to *H. virescens* (Cutler et al., 1986), there is no evidence of acute toxicity of *S. berthaultii* sucrose esters to *M. persicae*. Ingestion may be a requirement for toxicity. Aphids are able to avoid ingestion of these compounds because of their method of feeding. The probable cause of death is starvation. *M. persicae* suffers significant mortality after 72 hr of starvation, which is consistent with the

results of our experiments. Sucrose esters were not toxic to the green peach aphid during 48 hr of exposure in feeding cages. Aphids avoided the treated surface and did not feed. When caged on plants, aphids that eventually died were found off the leaf surface for one or more observations prior to death, and dead aphids were always found on the cage and not on the plant.

Despite the lack of toxicity, sucrose esters are effective deterrents of settling and probing by aphids. Our results suggest that contact with organs other than the stylets is required for behavioral activity. The behavioral activity of these compounds could be due to either chemosensory or tactile responses. Sucrose esters are tacky when applied to a parafilm membrane. Other tacky materials such as Tween 80 also inhibit aphid settling (Neal and Tingey, unpublished data; 4 mg/cm^2 reduced settling at 24 hr from 60% to 0%, $N = 10$). However, chemosensory-based avoidance of Tween 80 is also possible. Although little is known about the gustatory properties of sucrose esters from *S. berthaultii*, sucrose octaacetate, an ester used to denature alcohol, is intensely bitter to humans (Windholz et al., 1976) and may be perceived similarly by aphids.

While previous studies have demonstrated immobilization of aphids by type A exudate (Gibson and Turner, 1977), aphids were not immobilized in our study. Perhaps the design of the cage allowed the aphids a convenient exit from the leaf surface before they ruptured sufficient type A trichomes. Consistent with previous reports (Tingey and Laubengayer, 1981), the presence of sucrose ester droplets led to an increase in the number of tarsi that accumulated type A exudate.

King et al. (1987b) have shown that several tuber-bearing *Solanum* spp. possessing secretory type B trichomes accumulate sucrose esters as the primary component of trichome exudate. These species have also been described as insect resistant (Lapointe and Tingey, 1986; see also Tingey, 1981). Based on the physiological properties of both free fatty acids and their esters toward insects (Shepard, 1951), and on the biological activity of sucrose esters from *Nicotiana tabacum* (Cutler et al., 1986) and glucose esters of *L. pennellii* (Goffreda et al., 1988), it is likely that all the reported acyl sucroses contribute significantly to the aphid resistance reported in these species.

Investigation of the 2,3,4-tri-*O*-acyl glucose complex of *L. pennellii* trichomes indicates that individual glucose esters varying in carboxylic acid constituents do not differ in deterrent activity against potato aphid, *Macrosiphum euphorbiae*, in feeding cages (Goffreda et al., 1989). However, mixtures may be important in determining the viscosity or adhesive properties of the trichome droplets. Highly purified sucrose esters are less viscous than mixtures (personal observation). Water content or minor components of the droplets may also affect physical properties of the exudate. Optimum resistance to insects may depend on the physical characteristics of the droplet because proper adhesion to the

insect is required for optimum rupture of type A trichomes (Tingey and Laubengayer, 1981).

Commercial aphid-resistant potato cultivars bearing glandular trichomes containing sucrose esters may eventually be developed. Although commercial *S. tuberosum* cultivars lack the glandular trichomes necessary to deliver sucrose esters to the leaf surface, some *S. tuberosum* × *S. berthaultii* hybrids (Mehlenbacher et al., 1983) produce type B trichomes bearing droplets of sucrose esters (King et al., 1987b; Steffens et al., in preparation). In addition to deterring feeding by aphids, recent work indicates that 3,4,6-tri-*O*-acylsucrose contributes to late blight (*Phytophthora infestans*) resistance of *S. berthaultii* PI 473340 (Holley et al., 1987). Synthetic sucrose esters may also prove useful in foliar application for protection against aphids, aphid-borne diseases, and *P. infestans*.

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RESPONSE OF PARASITOID *Eucelatoria bryani* TO SELECTED PLANT MATERIAL IN AN OLFACTOMETER

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Abstract—The response of the parasitoid *Eucelatoria bryani* Sabrosky to fresh plant material and several plant extracts was examined in an olfactometer. The plants tested can serve as a food source for hosts (*Heliothis* spp.) of the parasitoid. Females responded positively to 13 of the 19 fresh plant tissues tested, and all of the extracts tested. Males responded to only two of the fresh plant tissues and to none of the extracts. The significance of plant variety, morphology, and phenology on the host habitat-selection behavior of this parasitoid is discussed.

Key Words—Behavior, biological control, *Eucelatoria bryani*, Diptera, Tachinidae, parasitoid, habitat selection, semiochemical, *Heliothis* spp., Lepidoptera, Noctuidae.

INTRODUCTION

Host habitat location is one of the critical first steps in the host selection behavior of many entomophagous insects (Vinson, 1976), many of which, particularly hymenopterous and dipterous parasitoids, will search extensively for hosts on some plants, while ignoring others. In some species, this searching has been shown to occur in habitats whether or not host insects are present

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(Vinson, 1981). In addition, some parasitoids may be attracted only to certain parts of the same plant (Varley, 1941; Smith, 1943). Thus, it is apparent that plant-produced chemicals are extremely important in the orientation and searching behavior of many entomophages.

Eucelatoria bryani Sabrosky is one of the most common tachinid parasitoids of larval *Heliothis* spp. in the southwestern United States (Bryan et al., 1972). Currently, an artificial diet is under development in the hope that *E. bryani* can be economically mass produced and used in inundative releases to control *Heliothis* spp. (Nettles et al., 1980; Nettles, 1986). In order to utilize this parasitoid effectively as a biological control agent, information concerning its host habitat-selection behavior is needed. Nettles (1979, 1980) has shown that *E. bryani* females are attracted to volatiles from cotton and okra plants. The present study was conducted to further elucidate the role of plant-produced semiochemicals in the host habitat-location behavior of *E. bryani*. This information should assist in determining the most appropriate times and locations for mass releases of this parasitoid.

METHODS AND MATERIALS

Insects. E. bryani were reared on cotton bollworm, *Heliothis zea* (Boddie), larvae as described by Nettles (1980). The rearing room was maintained at ca. 27°C, ambient relative humidity, and the flies were kept under a 14:10 hr light–dark photoperiod. Bollworm larvae were reared in large grids on a Nutrisoy diet (Shaver and Raulston, 1971) without corncob grits.

Plants. Most of the plant materials chosen for this study are listed as hosts of *H. zea* or *Heliothis virescens* (F.), or both, by Tietz (1972). Pigeonpea, *Cajanus cajans* (L.), and the unicorn plant, *Martynia louisianica* Mill., are not listed in Tietz (1972) as hosts for *Heliothis* spp.; however, flowering piogeonpea is extremely attractive to ovipositing *Heliothis* spp. (Lopez, 1982), and the unicorn plant was reported as a host for *Heliothis* spp. in the delta of Mississippi (Stadelbacher et al., 1986). In addition, the unicorn plant is frequently searched by *Cardiochiles nigriceps* Viereck, an important hymenopteran parasitoid of *H. virescens* (personal observation). Plant materials used in this study were carefully inspected for signs of *Heliothis* spp. infestation, briefly rinsed in distilled water, and blotted dry prior to their use. The plant materials tested against *E. bryani* are listed in Table 1.

Preparation of Extracts. Hexane extracts of pigeonpea flowers (two varieties), velvetleaf leaves, and sorghum head branchlets were prepared using the following procedure. Fresh material was soaked in hexane for 5 min, homogenized for 2 min in a Waring blender, and the resulting macerate filtered through Whatman No. 1 filter paper. The extracts were then evaporated to ca. 100 ml

TABLE 1. PLANT MATERIAL TESTED AGAINST PARASITOID *Eucelatoria bryani* IN AN OLFACTOMETER

Plant name	Variety	Source[a]	Tissue(s) screened
Cotton, *Gossypium hirsutum* L.	Stoneville 213	G	leaves flowers
Corn, *Zea mays* L.	Golden Cross Bantam	F	silks silk extract
Okra, *Hibiscus esculentus* L.	Louisiana Green Velvet	G	leaves
Pigeonpea, *Cajanus cajan* (L.) Millsp.	ICPL 131 (C11 line)[b]	F	leaves (tested early season) leaves (tested mid-season) leaves from flowering plants flower buds and flowers flower extract
	Norman[c]	F	leaves (tested early season) leaves from flowering plants flower buds and flowers flower extract
Sorghum, *Sorghum bicolor* (L.) Moench	Funk G-1602	F	head branchlets head branchlet extract
Tomato, *Lycopersicon esculentum* Mill.	Better Boy	G	leaves
Tobacco, *Nicotiana tabacum* L.	North Carolina 2326	G	leaves leaves from flowering plants flowers
Unicorn plant, *Martynia louisianica* Mill.		F	leaves flowers
Velvetleaf, *Abutilon theophrasti* Medik.		G	leaves leaf extract

[a] Plant material was obtained either from field- (F) or greenhouse- (G) grown plants.
[b] Indeterminate variety. Seed obtained from India.
[c] Determinate variety. Prior to our obtaining seed, this line had been selected for short season characteristics.

on a rotary evaporator, passed through anhydrous Na_2SO_4 crystals, and brought up to a concentration equivalent to 1 g plant material/ml hexane.

Parasitoids did not respond to hexane extracts of corn silks, but did respond to a water-soluble fraction of a chloroform–methanol extract. Fresh corn silks were soaked in a solution of chloroform–methanol (2 : 1) for 5 min, homogenized for 2 min in a Waring blender, and the resulting macerate was filtered through Whatman No. 1 filter paper into a large separatory funnel. The extract was then partitioned by vigorously mixing in a volume of distilled water,

approximately equal to that of the methanol, and allowing the extract to settle overnight at room temperature. Methanol from the methanol–water fraction was removed on a rotary evaporator, and the water-soluble component was concentrated to 1 g plant material/ml water. All extracts were stored in culture tubes at ca. −20°C until used.

Bioassay. Response of *E. bryani* to the plant material was tested in a Plexiglas olfactometer (99 × 30.5 × 50.8 cm) as described by Nettles (1980). During the test, each of the entry ports was fitted with two glass tubes (20 cm long, 5.1 cm ID) joined in series with duct tape. Each of the inner glass tubes (those closest to the olfactometer) was fitted with a flat aluminum screen at the outer end and a funnel-shaped screen (pointing inward) at the other end, so that flies entering the tube would be trapped. Both of the outer glass tubes were lined with heavy chromatography paper (Whatman, 3 MM) moistened with distilled water. Plant material (1–5 g) was placed in one of the outer tubes, while the other served as a control and contained only the moist filter paper. Air was passed through an activated charcoal filter and entered the glass tube arrangement at a velocity of ca. 1.2 m/sec. Air exited the opposite end of the olfactometer directly into the room through a large (25 × 45 cm) rectangular opening covered with a fine wire screen.

Plant extracts were applied to small (5.5 × 7.5 cm) sections of chromatography paper (Whatman, 3 MM), allowed to dry under a fume hood for ca. 10 min, then placed into the test chamber as described above. An equal volume of the appropriate solvent was applied to a second section of chromatography paper, allowed to air-dry, and placed in the control tube. Preliminary tests indicated that, depending on the extract used, different amounts were required to elicit a consistent response from the parasitoids. One gram equivalent was used for each assay of velvetleaf and both varieties of pigeonpea; 2-g equivalents of sorghum, and 5-g equivalents of the water-soluble corn silk extract were used for each test.

Since *E. bryani* are positively phototactic, the olfactometer was centered directly under the only light in the room. Illuminance at the top of the olfactometer was measured at 646 lux. In addition, a 28.5 × 46.5 cm section of white filter paper was placed on each side of the front end of the olfactometer, so that light intensity would be the same on both sides. Photoperiod, temperature, and humidity in the olfactometer room were kept the same as the rearing room, described above.

About 300–350 flies of both sexes, 7–11 days old, were released into the olfactometer at least 12 hr prior to the beginning of testing. Flies were provided with sugar cubes and moistened Cellucotton at all times. At the end of the 1-hr test period, flies that were caught in the trap tube were N_2-anesthetized, counted, sexed, and returned to the olfactometer. Between experiments, the glass tubes

were washed with a detergent solution, rinsed in acetone, and heated to 75°C for at least 2 hr.

We initially tried to conduct three to four experiments per day but found that the flies did not respond well under these conditions, possibly because of excessive handling and anesthetization. The best results were obtained when we conducted two runs per day in the olfactometer, one in the morning and one in the afternoon. Any bias that may have existed between the two entry ports was eliminated by alternating the port that received the treated and control tubes. Each material was tested against two or more separate groups of flies, and data are based on 10 replicates of each material. Data were analyzed using the *t* test procedure of SAS (SAS Institute, 1985).

RESULTS AND DISCUSSION

Overall, *E. bryani* females responded positively to the plant materials tested (Table 2). Positive responses to 13 of the 19 fresh plant tissues tested, and all five of the plant extracts were recorded. Males, on the other hand, responded positively only to fresh corn silks and leaves of velvetleaf. Curiously, none of the plant extracts, including those of these two plants elicited a positive response from males. Because of the taxonomic diversity of the plant materials tested, female flies may be responding to a general class of plant-produced chemicals.

Cotton was the only plant from which none of the tissues tested elicited a positive response from female flies. This is in contrast with Nettles (1980), who found that, under certain conditions, both male and female *E. bryani* responded to fresh cotton leaves. However, Nettles (1980) used the Deltapine 15 variety of cotton, and this discrepancy may be due to varietal differences in plant chemistry. Werner and Butler (1979) found that *Eucelatoria* sp. were one of the most abundant tachinid parasitoids collected in flight traps in an unidentified variety of short-staple cotton in Arizona. Franklin and Holdaway (1966) demonstrated that the tachinid parasitoid *Lydella grisescens* Robineau-Desvoidy is much more effective against the European corn borer, *Ostrinia nubilalis* (Hübner), on one corn hybrid than on another. Since *L. grisescens* is primarily attracted to plants of its host and secondarily to the host itself, this is likely a result of differences in plant-produced stimuli (Franklin and Holdaway, 1966).

Varietal differences in plant-produced chemicals have been shown in many plants, including cotton (Elzen et al., 1986). Generally, agronomic varieties are bred with characteristics such as high-yield, standability, quality, and resistance to disease and insects, with no thought given to effects on organisms in the third trophic level. Since plant-produced chemicals appear to play a vital role in the host selection process of many entomophagous insects (Nordlund et al., 1988),

TABLE 2. MEAN NUMBER (±SEM) OF *Eucelatoria bryani* RESPONDING PER HOUR TO VARIOUS PLANT TISSUES AND TISSUE EXTRACTS IN AN OLFACTOMETER[a]

Plant material	Females		Males	
	Treated tube	Control tube	Treated tube	Control tube
Cotton				
Leaves	5.9 ± 1.35	7.7 ± 1.75	7.2 ± 1.32	10.1 ± 1.37
Flowers	13.9 ± 2.87	12.8 ± 1.49	11.3 ± 1.52	12.7 ± 1.58
Corn				
Silks	14.6 ± 2.64	4.2 ± 1.14	19.1 ± 2.71	7.7 ± 1.13
Silk extract	12.3 ± 0.99	4.7 ± 0.70	9.9 ± 2.02	7.5 ± 0.78
Okra				
Leaves	6.0 ± 0.86	3.6 ± 0.67	4.3 ± 0.99	6.2 ± 1.12
Pigeonpea				
ICPL 131 variety				
Leaves (early)	11.6 ± 2.84	9.0 ± 2.25	12.1 ± 2.35	15.1 ± 2.10
Leaves (mid)	12.7 ± 1.19	2.7 ± 0.63	5.9 ± 2.85	6.7 ± 1.13
Leaves from flowering plants	8.8 ± 0.76	3.0 ± 0.65	6.2 ± 0.77	6.9 ± 0.97
Flower buds and flowers	19.2 ± 1.56	3.2 ± 0.65	6.7 ± 0.86	6.6 ± 0.91
Flower extract	11.2 ± 0.88	3.5 ± 0.54	8.8 ± 0.76	7.7 ± 1.06
Norman variety				
Leaves (early)	17.8 ± 2.36	7.1 ± 1.17	15.0 ± 1.64	13.5 ± 1.64
Leaves from flowering plants	32.3 ± 2.13	15.1 ± 1.38	20.1 ± 2.01	20.9 ± 2.02
Flower buds and flowers	21.5 ± 3.74	7.7 ± 1.40	12.6 ± 1.24	11.8 ± 1.36
Flower extract	22.2 ± 2.24	10.3 ± 1.26	20.2 ± 2.56	17.2 ± 2.17
Sorghum				
Head branchlets	17.3 ± 4.32	6.4 ± 1.33	19.6 ± 4.50	11.0 ± 1.50
Head branchlet extract	23.3 ± 1.94	11.5 ± 0.60	22.3 ± 2.30	19.8 ± 1.64
Tomato				
Leaves	10.0 ± 1.85	3.5 ± 0.91	9.3 ± 1.09	6.9 ± 1.43
Tobacco				
Leaves	7.4 ± 1.73	6.7 ± 1.07	7.9 ± 2.33	13.3 ± 3.41
Leaves from flowering plants	8.7 ± 0.79	7.7 ± 0.98	8.9 ± 0.87	10.7 ± 1.01
Flowers	13.7 ± 1.36	7.4 ± 1.37	7.3 ± 0.70	7.2 ± 0.89
Unicorn plant				
Leaves	7.7 ± 1.33	6.1 ± 1.53	8.1 ± 1.18	8.1 ± 1.46
Flowers	14.5 ± 1.99	6.6 ± 0.75	14.2 ± 2.37	12.7 ± 1.53
Velvetleaf				
Leaves	18.7 ± 2.97	7.2 ± 1.18	20.7 ± 2.57	11.2 ± 1.16
Leaf extract	14.9 ± 1.60	6.2 ± 0.95	10.7 ± 1.30	11.2 ± 1.21

[a] Means not attached by an underscore are significantly ($P < 0.05$) different as determined by t test.

the development of varieties that encourage and that are compatible with entomophagous insects should also be an important goal of plant breeders.

With the exception of early-season (ca. six weeks postplanting) leaves from ICPL 131, all tissues tested from both varieties of pigeonpea elicited a positive response from *E. bryani* females. In general, however, in terms of the number of flies responding, the Norman variety appears to be a slightly more attractive plant. Further, all tissues from Norman, including early-season leaves, were attractive to female flies (because Norman has been selected for short-season characteristics, plants were flowering at 12 weeks postplanting, which was the time interval established for testing mid-season leaves from ICPL 131). This is further evidence that varieties of the same plant species may affect parasitoid behavior in different ways.

In some of the plants tested, female *E. bryani* responded positively only to certain parts of the plant. Flies did not respond to leaves from nonflowering tobacco and unicorn plants, nor to leaves from flowering tobacco plants, but did respond to flowers from these plants. Although plant nectar is a component of the flies' natural diet, the positive response to these flowers was not likely food-oriented since: (1) only females responded in a positive manner, and (2) flies were provided sugar cubes and water at all times. Nettles (1979) reported that significantly more *E. bryani* females responded to okra flowers than to leaves of the same plant.

Similarly, flies did not respond to early-season (ca. six weeks postplanting) leaves of pigeonpea (ICPL 131), but leaves from more mature plants (ca. 12 weeks post-planting) did elicit a positive response. Thus, response by this parasitoid to plant material is dependent not only on the plant tissue in question but also on the age of that plant tissue. These may be general mechanisms by which the parasitoid is synchronized with its host population on certain host plants. Although there are undoubtedly many components involved in the synchronization between host and parasitoid, it is likely that plant phenology plays a role in this complex interaction. The life cycles of the tachinid parasitoid *Eucarcelia rutilla* Vill., and its host, *Bupalus piniarius* L., are so closely synchronized that the parasitoid almost exclusively attacks caterpillars feeding on pine needles between mid-July and the end of August (Herrebout, 1969).

Results from this study suggest that *E. bryani* may be a useful agent for control of *Heliothis* spp. in sorghum. Fresh sorghum head branchlets as well as extracted material elicited a strong response from female flies. Sorghum has also been shown to be highly attractive to *Campoletis sonorensis* (Cameron), an ichneumonid parasitoid of *Heliothis* spp. (Elzen et al., 1983). Because *Heliothis* spp. larvae are particularly vulnerable to parasitoid attack in open-headed sorghums (Young and Teetes, 1977), it is possible that one or both of these entomophagous agents could effectively control this pest.

E. bryani females also responded to fresh corn silks and a water-soluble corn silk extract. This attraction to corn has been observed in Arizona, where adult *E. bryani* are frequently collected via sweep net in corn fields (C. G. Jackson, personal communication). Biological control of *Heliothis* spp. larvae in silking corn is somewhat difficult because the majority of larval feeding occurs within the ear and thus, the larvae are rarely exposed. Because *E. bryani* is highly attracted to corn, it may prove to be important in the control of *H. zea* in this crop in certain geographic areas. Another tachinid, *Archytas marmoratus* (Townsend), is a major parasitoid of large *H. zea* larvae in corn grown in the southeastern United States (Gross and Young, 1984).

It is interesting to note that *E. bryani* were attracted to flowers and/or leaves of the two weed species that we tested. *Heliothis* spp. will oviposit heavily on velvetleaf in central Texas (personal observation), and has been reported to be a good host plant for *Heliothis* spp. development in the delta (flood plain) region of Mississippi (Stadelbacher, 1981). Although we never observed *Heliothis* spp. on unicorn plant in central Texas, it has been reported as a host of *H. virescens* in Mississippi, albeit a poor one (Stadelbacher et al., 1986). Weeds are a normal component of agroecosystems and have been shown to be important in the buildup of early-season *Heliothis* spp. populations (Stadelbacher et al., 1986). In addition, it has been suggested that these early-season populations of *Heliothis* spp. could be suppressed via habitat management and augmentative releases of predators and parasites (Stadelbacher et al., 1986). In certain areas, *E. bryani* may prove to be one of these parasites.

The data presented here indicate that female *E. bryani* are attracted to volatile chemicals from certain plants and that these chemicals may be important cues in the host habitat selection behavior of this parasitoid. This study also shows that response by this parasitoid can be affected by plant variety, plant morphology, and plant phenology. These factors likely influence the behavior of many parasitoids. Elzen et al. (1986) found that the parasitoid *C. sonorensis* exhibited a greater response to glanded cotton cultivars than glandless cultivars. In addition, there may be other factors, such as geographic variability, that influence host–parasitoid interactions. Lanier et al. (1972) found that the parasitoid *Tomicobia tibialis* preferred the kairomone produced by *Ips pini* (Say) from California and Idaho to that produced by beetles from New York. Herbivore defense against natural enemies can also vary geographically (Price, 1981). This variability in parasitoid behavior should be considered when exploring for new biological control agents and when evaluating the performance of these agents under specific conditions.

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INTERPOPULATIONAL VARIATION IN EMITTED PHEROMONE BLEND OF CABBAGE LOOPER MOTH, *Trichoplusia ni*

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Abstract—Female cabbage looper moths, *Trichoplusia ni*, from laboratory colonies initiated from three locations across the United States emitted similar quantities and blend ratios of the six known pheromone components. In contrast, females from a long-established laboratory colony emitted a greater proportion of four of the five minor components relative to the major component, (*Z*)-7-dodecenyl acetate; only the relative proportion of 11-dodecenyl acetate was similar in all of the populations sampled. Females from this population emitted (*Z*)-7-dodecenyl acetate at a rate similar to that from females from field-collected colonies. Within each population there were highly significant correlations among the quantities of pheromone components of similar molecular weights. Correlations between components of different molecular weights were not as great, but often were significant. Similarities of blend ratios among field populations may indicate that the chemical signal in this species is conservative. The difference of the blend ratios in our laboratory population from the other populations may indicate a decrease in the intensity of selection pressure that usually would maintain these values.

Key Words—Sex pheromone, (*Z*)-7-dodecenyl acetate, (*Z*)-5-dodecenyl acetate, 11-dodecenyl acetate, dodecyl acetate, (*Z*)-9-tetradecenyl acetate, (*Z*)-7-tetradecenyl acetate, Lepidoptera, Noctuidae, *Trichoplusia ni*.

INTRODUCTION

Studies of communication systems of insects have sometimes documented genetically based geographical variation in the emitted blend of pheromone components within certain species (e.g., European corn borer, *Ostrinia nu-*

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bilalis, Klun and Cooperators, 1975; *Ips pini*, Lanier et al., 1972; European turnip moth, *Agrotis segetum*, Lofstedt et al., 1986). Such divergence may be explained by directional selection and/or genetic drift. In contrast to these cases, the pheromone blend emitted by some species maybe fairly constant throughout their geographic range (e.g., Haynes and Baker, 1988). This consistency may be favored by gene flow between populations and mutually imposed stabilizing selection between signaler and responder (e.g., Ryan and Wilczynski, 1988).

When populations are maintained in continuous culture under laboratory conditions, these evolutionary forces may be quite different. Gene flow between a laboratory population and other populations is usually absent. Genetic drift resulting from founder effects and genetic bottlenecks may be more likely. Potential selection on the communication system imposed by heterospecifics is eliminated. The close proximity of many males and females may reduce the potential stabilizing selection imposed by signalers on responders and responders on signalers.

The sex pheromone of the cabbage looper moth, *Trichoplusia ni*, was identified as (Z)-7-dodecenyl acetate (Z7–12 : Ac) (Berger, 1966), but is now known to consist of this major component and dodecyl acetate (12 : Ac), (Z)-5-dodecenyl acetate (Z5–12 : Ac), 11-dodecenyl acetate (11–12 : Ac), (Z)-7-tetradecenyl acetate (Z7–14 : Ac), and (Z)-9-tetradecenyl acetate (Z9–14 : Ac) (Bjostad et al., 1984; Linn et al., 1984). We investigated interindividual and interpopulational variation in the pheromone blend emitted from sex pheromone glands in this species. Included in our analysis was a colony of *T. ni* that has been maintained in culture for over 100 generations without the reintroduction of insects from field-collected populations.

METHODS AND MATERIALS

Adult *T. ni* were collected in the field by using inverted cone traps (Scentry) that were baited with phenylacetaldehyde (a presumed floral attractant). Collection sites were in Riverside, California; Lexington, Kentucky; and Gainesville, Florida. At least 40 individuals were trapped at each collection site over the course of several weeks. Collected males and females were placed together in 1-gal cartons and females oviposited on paper towels. Eggs laid by females from California and Florida were sent by overnight mail to Kentucky where separately maintained colonies were established. Thereafter, rearing procedures followed the same protocol as those used for the laboratory colony that originated from populations near Riverside, and has been maintained using the procedures of Shorey and Hale (1965). Pupae were separated according to sex. Female pupae were housed in 1-pint (473-ml) paper cartons with nylon-screen lids. Emerged adults were removed daily and transferred to additional cartons.

Just prior to the onset of scotophase, when females were 3–5 days old, they were placed individually in 4 cm diameter × 4 cm height hardware cloth (3-mm mesh) cages closed with aluminum lids.

Collection of pheromone from females followed the procedures described by Baker et al. (1981), except that intact females were used. During the second through eighth hour of the scotophase, a period during which calling behavior was observed under the experimental conditions, a female was removed from an environmental chamber and inserted abdomen first into a glass female-holder. The holder was 5.5 cm long, 0.6 cm in diameter, and had a ca. 1-mm hole at the distal end that allowed the ovipositor and associated sex pheromone gland to extrude out of the holder when pressure was applied to the head of the female with a pipe cleaner. Females could then be removed from the holder and usually would survive for several days. The holder was then inserted into the all-glass collector described by Haynes et al. (1984). A stream of nitrogen gas flowed through the collector at a rate of 120 ml/min. After the 10-min collection period, 10 ng of an internal standard [(*E*,*Z*)-4,7-tridecadienyl acetate] was added to the adsorbent glass wool surface at the distal end of the collector which was then rinsed with two 50-μl aliquots of CS_2. This volume was reduced to ca. 1 μl before it was injected into a 30 m Carbowax 20 M capillary column in a Hewlett-Packard 5890A GC linked to a Hewlett-Packard 5970B Mass Selective Detector. The sensitivity of this instrument was optimized by manual tuning on ion 69 (*m*/*z*) of the tuning standard perfluorotributylamine, and subsequently using selective ion monitoring of ions 54, 55, and 67 (*m*/*z*), which are abundant in all of the reported pheromone components. Standard curves that related peak area (*m*/*z* 54 + 55 + 67) to the mass of each pheromone component were established because the relative abundance of these ions was not the same for all molecules of interest. The lower analytical limit using this technique was ca. 0.01 ng for *Z*9–14 : Ac (the least abundant component).

To determine the relative accuracy of the emission rates and blend ratio obtained using the extruded gland technique, a comparison was made to collections from free-calling females. A disposable pipet (Gold Seal No. 4642) was cut 1 cm before it starts to taper and 2 cm from the tip of the stem, thus leaving a 4-cm funnel-shaped collector. The stem of the collector was packed with 10 mg of glass wool. When a female began to call, the narrow end of this funnel collector was connected to a regulated vacuum system that allowed 120 ml/min to flow through the system. The collector was then positioned so that the sex pheromone gland of the female was just within the wide end of the funnel, but did not touch any of the glass surface. The females usually were not disturbed by this procedure and generally continued to call for at least 10 min, at which time the collection was terminated. Collections from these free-calling females alternated with collection from females that had begun calling, but were immediately inserted into the other collection system. The use of an internal standard

and the procedures for extracting pheromone components were the same as for the extruded gland technique.

RESULTS

The volatile collection technique allowed us to determine both the emission rate and blend ratio emitted by individual females (Figure 1). There were no significant differences in the females' emission rate of

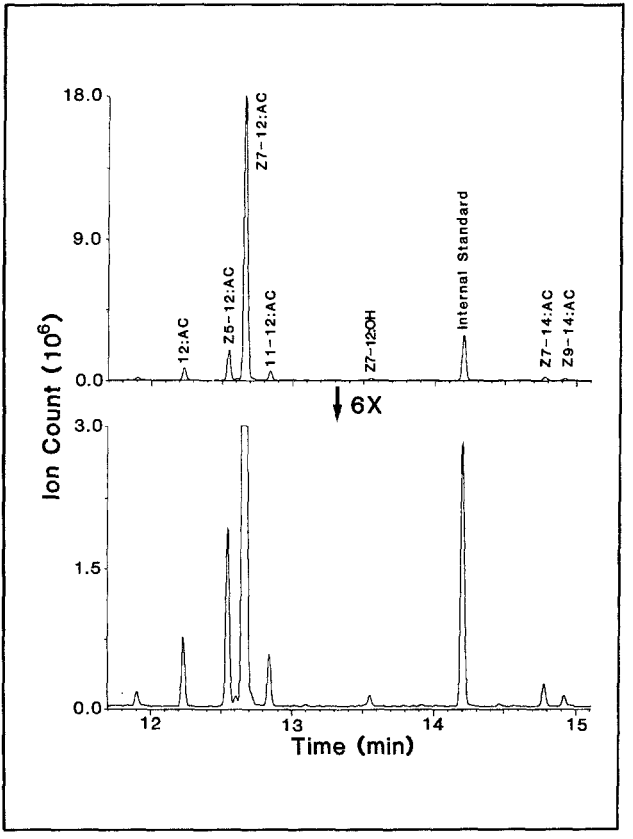

FIG. 1. Chromatogram obtained from the analysis of the compounds emitted from a single laboratory female. The lower trace is identical to the upper trace but is amplified sixfold to show the minor components. An internal standard [10 ng of (*E*,*Z*)-4,7-tridecadienyl acetate] was added to the collector before it was rinsed with carbon disulfide.

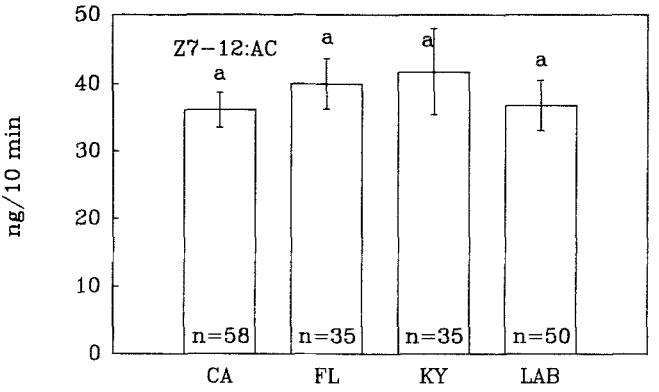

FIG. 2. Mean quantity (± SEM) of (Z)-7-dodecenyl acetate emitted during 10 min collection periods. There was no significant difference between populations of females ($P > 0.05$, one-way analysis of variance).

Z7-12:Ac, the major component, among the four populations sampled (Figure 2; one-way analysis of variance; $P > 0.05$). The low mean emission rate was 36.2 ng/10 min from females originating from the California colony, and the high mean emission rate was 41.8 ng/10 min from females originating in Kentucky. The measured emission rate of Z7-12:Ac was variable; for example, in females from the laboratory population it ranged from 1.26 ng/10 min to 124.1 ng/10 min.

The proportions of the minor pheromone components from volatile emissions were fairly consistent among females from the three field-originating populations, but their mean proportions of Z5-12:Ac, 12:Ac, Z7-14:Ac, and Z9-14:Ac were significantly lower than those of females from the laboratory population (Figure 3; one-way analysis of variance, Student-Newman Keuls multiple comparison test; $P < 0.05$). Z7-12:OH was detected in every volatile collection but was less abundant in the emitted volatiles of females from the laboratory population ($P < 0.05$).

In general the correlation between the quantities of the six pheromone components compared two at a time showed a significant positive relationship (Table 1). Exceptions were found within the Florida populations in which the relationships between some of the 12-carbon acetates and 14-carbon acetates were not significant. Within all four populations the correlations were greater with molecules of similar molecular weight (e.g., quantities of Z7-14:Ac and Z9-14:Ac were more highly correlated than Z7-12:Ac and Z9-14:Ac).

Collections from free-calling females and females with forced gland extrusions demonstrated that the techniques yielded similar results, with two excep-

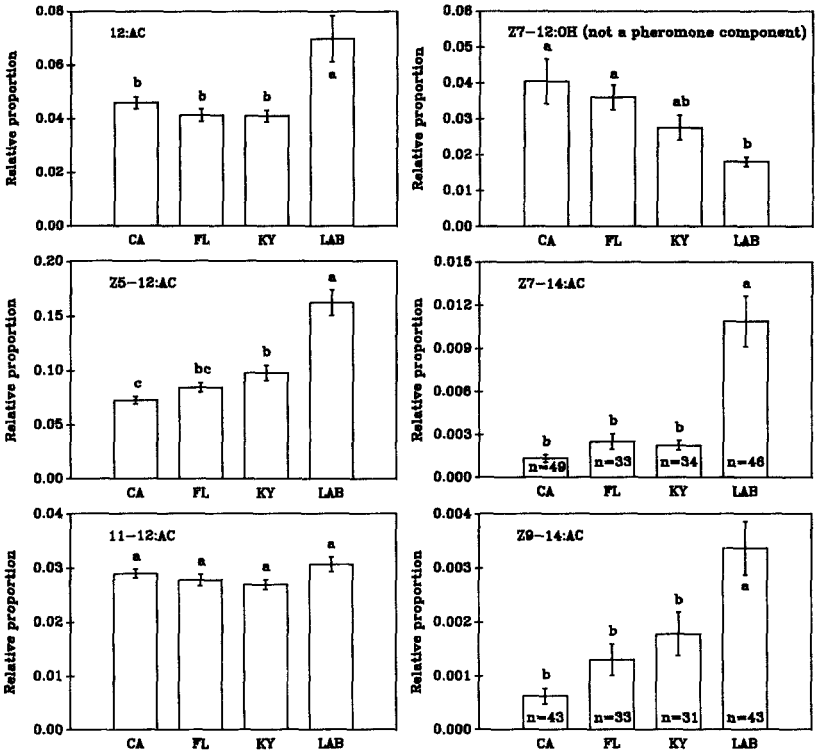

FIG. 3. Mean relative proportions (±SEM) of five minor pheromone components and (Z)-7-dodecenol emitted during 10-min collection periods. Number of individuals included for each mean was the same as in Figure 2, except where otherwise indicated. Means are significantly different when they do not share a letter in common ($P < 0.05$, one-way analysis of variance; Student-Newman-Keuls test for multiple comparisons).

tions (Table 2). First the emission rate from free-calling females was ca. 1.5 times higher than that measured from forced gland-extrusion collections (analysis of variance, $P < 0.05$). Second, no Z7-12:OH was detected from free-calling females.

DISCUSSION

The technique of obtaining volatile collections from forcibly extruded sex pheromone glands has proven to be an effective aid in identifying sex pheromone components (e.g., Bjostad et al., 1984; Dunkelblum et al., 1987). By allowing effective analysis of the blend emitted by individuals, these collections

TABLE 1. MATRIX DEPICTING CORRELATIONS (r VALUES) BETWEEN COMPOUNDS EMITTED FROM SEX PHEROMONE GLANDS OF *Trichoplusia ni*[a]

Compound 1	Compound 2	California (N = 39)	Florida (N = 32)	Kentucky (N = 31)	Lab (N = 41)
Z7-12:Ac	12:Ac	0.79[b]	0.86[b]	0.92[b]	0.91[b]
Z7-12:Ac	Z5-12:Ac	0.78[b]	0.91[b]	0.93[b]	0.77[b]
Z7-12:Ac	11-12:Ac	0.87[b]	0.93[b]	0.98[b]	0.95[b]
Z7-12:Ac	Z7-14:Ac	0.33[b]	0.35[b]	0.78[b]	0.37[b]
Z7-12:Ac	Z9-14:Ac	0.34[b]	0.41[b]	0.74[b]	0.39[b]
12:Ac	Z5-12:Ac	0.72[b]	0.83[b]	0.88[b]	0.71[b]
12:Ac	11-12:Ac	0.93[b]	0.95[b]	0.97[b]	0.94[b]
12:Ac	Z7-14:Ac	0.48[b]	0.17	0.72[b]	0.42[b]
12:Ac	Z9-14:Ac	0.51[b]	0.19	0.70[b]	0.45[b]
Z5-12:Ac	11-12:Ac	0.77[b]	0.85[b]	0.92[b]	0.65[b]
Z5-12:Ac	Z7-14:Ac	0.61[b]	0.41[b]	0.84[b]	0.51[b]
Z5-12:Ac	Z9-14:Ac	0.53[b]	0.47[b]	0.74[b]	0.37[b]
11-12:Ac	Z7-14:Ac	0.45[b]	0.16	0.76[b]	0.32[b]
11-12:Ac	Z9-14:Ac	0.46[b]	0.21	0.72[b]	0.37[b]
Z7-14:Ac	Z9-14:Ac	0.93[b]	0.90[b]	0.91[b]	0.91[b]

[a] Only females for which all six components could be accurately quantified were included.
[b] Significant positive correlation between emission rate of compound 1 and compound 2.

TABLE 2. COMPARISON OF TWO METHODS OF COLLECTING VOLATILIZED PHEROMONE FROM FEMALE *Trichoplusia ni*

Compound	Type of volatile collection[a]	
	Free-calling (N = 10)	Gland extrusion (N = 10)
Quantity		
Z7-12:Ac (ng/10 min)	65.1 ± 6.68a	44.6 ± 3.19b
Relative proportion[b]		
12:Ac	0.066 ± 0.0045a	0.067 ± 0.0041a
Z5-12:Ac	0.126 ± 0.010a	0.131 ± 0.010a
11-12:Ac	0.040 ± 0.0023a	0.035 ± 0.0024a
Z7-12:OH	0[c]b	0.016 ± 0.0017a
Z7-14:Ac	0.0079 ± 0.0027a	0.0044 ± 0.0008a
Z9-14:Ac	0.0030 ± 0.0009a	0.0023 ± 0.0010a

[a] Means in the same row are significantly different if followed by different letters ($P < 0.05$, Kruskal-Wallis one-way analysis of variance).
[b] Relative proportions were calculated by dividing the quantity of each component by the quantity of Z7-12:Ac.
[c] Z7-12:OH was absent or present below the limits of detection of this analysis.

have enabled us to gain a more realistic impression of interindividual variation and populational variation in pheromone blends (e.g., Haynes et al., 1984; Haynes and Baker, 1988). One assumption underlying the technique that we used in this study is that the moth emits the same quantity and quality of pheromone when the gland is forcibly extruded as when calling naturally. In moths that actively pump their sex pheromone glands, females may emit ca. 25 times more pheromone than would be indicated by forced collections (Schal and Cardé, 1985). Krasnoff and Roelofs (1988) documented that rhythmic protrusion and retraction of the pheromone gland by female *Pyrrharctia isabella* results in an extraordinarily high emission rate of its pheromone in aerosol form. The cabbage looper moth everts its pheromone gland for longer periods of time (Bjostad et al., 1980), and thus there appears to be no repetitive retraction and eversion of the gland. There was a predictable relationship between both the quantity and quality of the pheromone blend emitted by females as measured by free-calling and forced-extrusion techniques. Females were measured emitting only ca. 1.5 times more Z7–12 : Ac during calling compared to collections from forced extrusion of glands. However, using the forced-extrusion technique, we detected significant quantities of Z7–12 : OH, which is a potent interruptor of the males' behavioral response to sex pheromone (McLaughlin et al., 1974; Tumlinson et al., 1972). Emission of Z7–12 : OH has been noted earlier by Bjostad et al. (1984) and was considered at artifact of the technique. In another species, the tomato looper, *Plusia chalcites*, Z7–12 : OH was detected using the volatile collection technique. This compound has been found to interfere with the behavioral response of male *P. chalcites*. Dunkelblum et al. (1987) suggested that small amounts of Z7–12 : OH could be produced by the hydrolysis of Z7–12 : Ac by other tissues from the female. This explanation could be valid for *T. ni* since Z7–12 : OH is apparently not produced by free-calling females. The forced-extrusion technique appears to yield valid information on the blend ratio of pheromone components and a good approximation of the emission rate.

The technique of obtaining volatile collections from extruded glands has allowed us to compare pheromone emission by females from populations originating from three localities distributed across the United States and from a long-maintained laboratory population. The populations originating from these localities were very similar in both the quality and quantity of the pheromone components. *T. ni* is a very mobile insect, is subtropical in origin, and has no winter diapause (Lingren et al., 1979). In areas such as southern California and Florida populations may persist year-round, but in more northern states (such as Kentucky in this study) spring immigration is responsible for initiating the local populations. Prevailing weather patterns suggest that overwintering populations in Louisiana and Texas are likely sources for populations that later

establish in Kentucky (Riordan, 1979). Lingren et al. (1979) suggested that a fall southerly migration occurs in this species. Such mobility suggests that some gene flow between the three populations is possible and may be sufficient to prevent the establishment of geographically isolated populations in which divergence in the chemical signal might occur, as in the acoustical signals of cricket frogs (Ryan and Wilczynski, 1988). However, in another species of moth, *Pectinophora gossypiella*, Haynes and Baker (1988) have established that the pheromone blend was fairly consistent throughout the worldwide populations that were sampled. The blend emitted by one population from China was significantly different from all other populations sampled (Argentina, Brazil, Egypt, Pakistan, and the United States), but the difference was very small. Thus geographical isolation alone does not necessarily lead to divergence in the pheromone blend.

Given the apparent conservative nature of the pheromone blend in field populations of cabbage looper moths, an important question is why the ratio in laboratory populations was different. A simple explanation is that the individuals used to initiate the laboratory population were not representative of the population from which they were removed—a founder effect. Alternatively, the laboratory population may have gone through severe population crashes, which then could have led to a shift in the blend ratios—genetic bottlenecks. There were extreme individuals in the field populations that emitted blends similar to those produced by laboratory females. Normally one might expect that selection pressure for precise control of the pheromone blend might remove those individuals that deviated from the norm. In laboratory populations this selection pressure may be much less intense, because long-range mate location is no longer a prerequisite for mating, and selection for specificity in the pheromone blend is no longer critical when other species are not in the same environment.

A similar deviation in the pheromone blend between laboratory and field population was demonstrated in *Argyrotaenia velutinana* (Miller and Roelofs, 1980). Females from a field-collected population emitted 9.1% (*E*)-11-tetradecenyl acetate to (*Z*)-11-tetradecenyl acetate, whereas the laboratory females emitted a 7.0% *E*–*Z* ratio. Again, in this species, it was not possible to determine whether the change was due to directional selection in the laboratory population or genetic drift.

If this hypothesis of random change is correct, one would expect that blend ratios of females from other laboratory populations to be different from our laboratory population. If predictable directional selection is responsible for the divergence of the pheromone blend in laboratory and field populations, then all laboratory populations maintained in a similar way to ours might show similar differences. We cannot answer this question because only one laboratory population has been sampled.

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A KAIROMONE FOR *Trichogramma nubilale* (HYMENOPTERA: TRICHOGRAMMATIDAE) Isolation, Identification, and Synthesis[1]

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Abstract—A kairomone that effects host-seeking behavior in *Trichogramma nubilale* Ertle and Davis, an egg parasitoid of the European corn borer, *Ostrinia nubilalis* (Hübner), was isolated from moth scales of the European corn borer. The kairomone was identified as a mixture of 11,15-, 13,17-, and 15,19-dimethylnonatriacontanes. The three dimethylnonatriacontanes were synthesized, and bioassays showed that the 13,17 isomer was the most active in terms of klinokinetic and retention effects. The 11,15 isomer and the 15,19 isomer had some effect on klinokinesis, but they failed to effect retention of the wasps. The 13,17-dimethylnonatriacontane is considered to be the most important component of the kairomone.

Key Words—Behavior, hydrocarbons, kairomone, kinesis, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, *Trichogramma nubilale*, Hymenoptera, Trichogrammatidae.

INTRODUCTION

Trichogramma nubilale Ertle & Davis is an important egg parasitoid of European corn borer (ECB), *Ostrinia nubilalis* (Hübner). In a Delaware study, parasitism of ECB egg masses on sweet corn ranged from ca. 5% in June to ca. 70% in September and, of more importance, 100% of the eggs are parasitized in ca. 90% of the parasitized egg masses (Ertle and Davis, 1975).

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Kairomones play crucial roles in host-finding behavior of *Trichogramma* spp. Lewis et al. (1972) found that moth scales of female *Heliothis zea* (Boddie) were the source of a kairomone that elicited a host-finding response from female *Trichogramma evanescens* Westwood and that the parasitism rate of *H. zea* could be increased by spraying crops with the kairomones. Jones et al. (1973) identified tricosane as the most active of several active hydrocarbons. Other host-associated chemicals, such a sex pheromones and organic acids, also have been identified as kairomones for *Trichogramma* spp. (Lewis et al., 1982; Gueldner et al., 1984).

Shu and Jones (1989) demonstrated that kairomones in the scales of ECB adults could affect the klinokinetic and orthokinetic behavior of *T. nubilale* in a manner that retained the wasps in a kairomone-treated area and resulted in an increase in parasitism rates of ECB eggs and egg masses. The present paper reports the isolation, identification, and synthesis of the chemicals responsible for this activity.

METHODS AND MATERIALS

Insects and Bioassay. The rearing and handling of *T. nubilale* and procedures for bioassays were described by Shu and Jones (1989). The bioassay consisted of the measurement of female responses to a chemically treated 4.5-cm-diameter circle on filter paper. The subject chemicals were applied at rates equivalent to 5 μg of moth scales in a volume of 50 μl (100 ng/μl) (Shu and Jones, 1989). Observation was through a glass plate on which the movement of a female, after release in the center of the circle, was traced. A value of 0 was assigned when walking behavior was the same in the treated circle as in the clean area outside the circle. Values up to 10 were assigned quantitatively as the time (based on the paths drawn) allotted to the treated circle increased beyond the clean area outside the circle. There were 15 replications for each treatment. In the bioassay of the synthetic chemicals, the number of turns in the treated circle was recorded with a digital counter, and time spent inside and outside the treated circle was recorded with a cassette tape recorder in a way that an "in" or "out" voice indicated that the female was inside the treated circle or outside. The recorded tape was played back to quantify the time. Percentage of time inside the treated circle and turning rates (turns/min) inside the treated circle were calculated. The bioassay of synthetic materials was designed as a randomized-block design and five treatments were randomly assigned to each of nine blocks. A block was one day. The five treatments were: MSE (moth scales extract), 11,15-DMC39 (DMC39 = dimethylnonatriacontane), 13,17-DMC39, 15,19-DMC39, and control (hexane only). Concentration of each synthetic chemical was 50 pg/μl, the approximate concentration of the

active hydrocarbons in the 100 ng/μl moth scale extract. A treatment volume of 50 μl deposited 2.5 ng of synthetic chemical per treatment. The data were analyzed by analysis of variance followed by a check of model fitness and the multiple-range test by Duncan (1955).

Collection and Extraction. ECB moth scales (1.5 g) were collected from laboratory-reared moths after holding them at 0°C for immobilization. The immobilized moths were shaken in a jar to remove scales, which were collected with a hand vacuum cleaner. The collected scales were Soxhlet extracted for 6 hr with 100 ml nanograde hexane that subsequently was concentrated to 60 ml on a rotary flask evaporator.

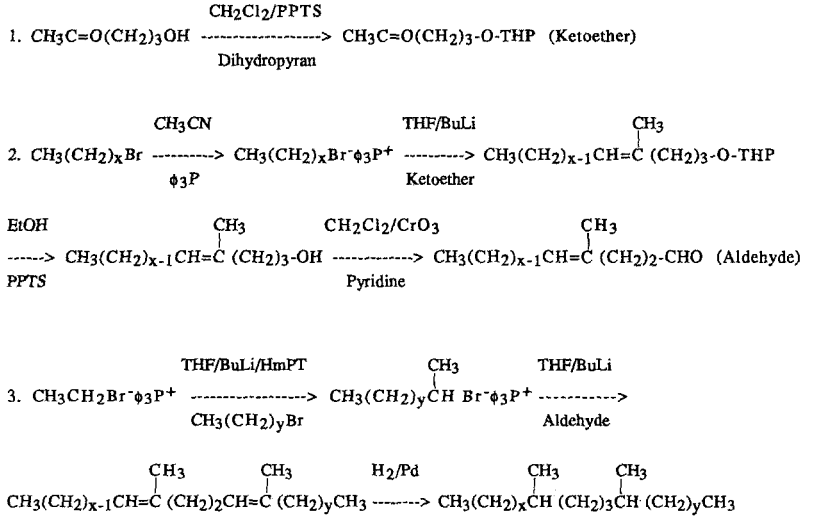
Isolation and Identification. A 50-ml aliquot of the 60-ml concentrated hexane extract was reduced to 1 ml under nitrogen and subjected to column chromatography on a 1.5 × 18-cm column containing Florisil (2% water by weight). The column was eluted successively with three void volumes (10 ml each) of hexane and two void volumes of: ether–hexane (2.5:97.5), ether–hexane (5:95), ether–hexane (7.5:92.5), ether–hexane (10:90), ether–hexane (25:75), ether-hexane (50:50), ether, acetone, and methanol. The 10 fractions were stored at −70°C.

Saturated and unsaturated hydrocarbons were separated by percolating the Florisil column hexane fraction through a 20% silver nitrate Florisil column (0.5 × 4 cm) with hexane to obtain the paraffins, followed by 2.5% ether in hexane to obtain the olefins. Straight-chain and branched hydrocarbons were separated with 5 Å molecular sieve (Hutchins and Martin, 1968).

Gas chromatography (GC) was performed with a 5830A Hewlett Packard GC equipped with a flame ionization detector (FID), effluent splitter, and thermal gradient collector (similar to that described by Brownlee and Silverstein, 1968). An all-glass column 1.9 m × 2 mm ID was packed with 3% Dexsil 300 on 80/100 Gas Chrom Q. The carrier gas was nitrogen at 20 ml/min. The temperature was programmed from 150° to 325°C with the first 12 min ramped at 8°C/min and thereafter at 3°C/min. Capillary gas chromatography was performed with a 5890 Hewlett Packard GC equipped with an FID and an effluent splitter that vented through a thermal conductivity detector (TCD) for collection. A glass capillary was connected to the TCD and cooled with Dry Ice. The column was a 0.53-mm × 30-m DB-5 column programmed to run isothermally at 300°C. The carrier gas was helium at 2.5 ml/min with a linear flow rate of 30 cm/sec and a column head pressure of 4.5 psig. All collected materials were rechromatographed to confirm purity and quantity.

Mass spectra were obtained on a Kratos GC-MS 25 equipped with a 0.53-mm × 15-m DB-5 column run at 290°C isothermal and using helium as a carrier gas at the rate of 3.5 ml/min. The mass spectrometer was operated at an ionization energy of 20 eV or 70 eV.

Synthesis. The synthetic route is outlined in Figure 1. All starting materials



Compound I. x=9, y=23. Compound II. x=11, y=21. Compound III. x=13, y=19.
PPTS=Pyridinium *p*-toluenesulfonate, THP=Tetrahydropyran, THF=Tetrahydrofuran, BuLi=n-butyllithium, HmPT=Hexamethylphosphoric triamide, ϕ_3P=triphenylphosphine.

FIG. 1. Synthesis route of three dimethylnonatriacontanes.

were commercially available except 1-bromotetracosane, which was synthesized from 1,12-dibromododecane and dodecyl aldehyde through a Wittig condensation. In all of the work we were guided by Sonnet (1976), Miyashita et al. (1977) for protection and deprotection of the hydroxy group of 5-hydroxy-2-pentanone, and Ratcliffe and Rodehorst (1970) for oxidation of the primary alcohol to the aldehyde. Synthetic materials for bioassay were purified on the 5830A GC described above. Collected materials were rechromatographed to check for purity and quantified by comparison with an internal standard.

RESULTS AND DISCUSSION

Isolation and Identification. The hexane Florisil fraction derived from the hexane extract of moth scales scored 8.3 of 10 in the bioassay. This was significantly higher than all other Florisil fractions (3.3) and the control (2.4).

Argentation chromatography of the active hexane Florisil fraction indicated the bioassay activity to be in the saturated hydrocarbon fraction. In addition, when the active Florisil fraction was subjected to 5 Å molecular sieve,

the excluded fraction retained activity indicating that the active chemicals were branched.

The active Florisil fraction was subjected to GC (3% Dexsil 300 column) analysis, and the effluent was collected in four fractions labeled A, B, C, and D from the first eluted GC peak to the last. Fraction C contained one peak and the other fractions contained more than one peak. Fraction C scored 8.0 of 10, while A, B, and D scored 2.4, 3.3, and 1.9, respectively. The control scored 2.1. The score of fraction C was significantly higher than the other fractions.

Fraction C was further subjected to GC (DB-5 column) analysis and showed the presence of four peaks labeled P-39A, 39B, 39C, and 39D. P-39A was not collected because it was a relatively small peak. P-39C and P-39D were incompletely resolved, so they were collected as one fraction. P-39B was collected in one fraction, and it scored significantly higher (7.9) than P-39C and P-39D (2.9) and the control (2.2).

The collected material in P-39B was rechromatographed with two internal standards, *n*-octatriacontane and *n*-tetracontane and was found to have an equivalent chain length of 39.5 (Miwa, 1963). The 39.5 equivalent chain length indicated that the compound(s) in P-39B could be dimethylnonatriacontane(s) (DMC39) (Nelson, 1978).

Sequential mass spectra during elution of P-39B demonstrated the presence of three components. Shown in Figures 2–4 are three 15× amplified spectra of P-39B taken at about 12 scans apart. These spectra had six odd mass peaks from secondary fragmentation [C_xH_{2x+1}-$(CH_3)CH$] at *m/e* 295, 379; 267, 407; and 239, 435, which were more intense than their preceding peaks of 14 mass units less. These peaks were also greater than the corresponding even mass peaks from secondary fragmentation with a hydrogen being transferred [C_xH_{2x}-$(CH_3)CH$] at *m/e* 294, 378; 266, 406; and 238, 434, an indication that the P-39B hydrocarbons were not internally branched monomethyl-alkanes (McCarthy et al., 1968; Nelson et al., 1972).

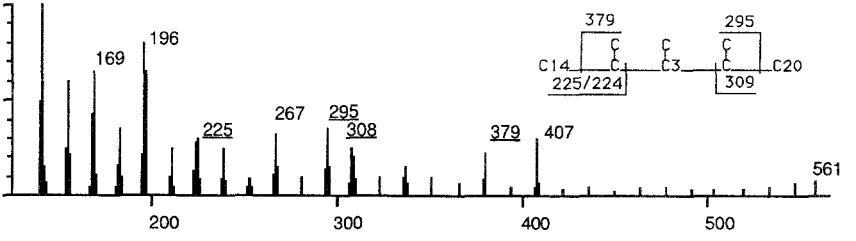


FIG. 2. Mass spectrum of a scan at 12 scans before the most intense scan with a mass range from 140 to 561: 15,19-dimethylnonatriacontane (most plausible structure). Ordinate, intensity; abscissa, *m/e*.

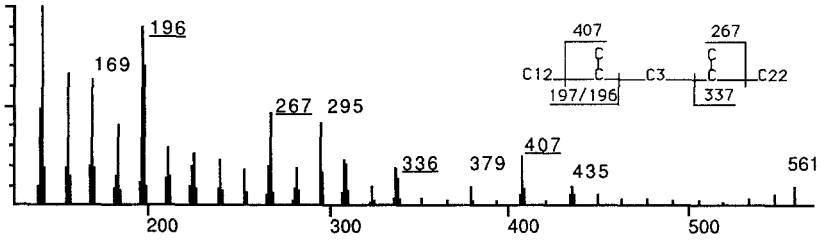


FIG. 3. Mass spectrum of the most intense scan with a mass range from 140 to 561: 13,17-dimethylnonatriacontane (most plausible structure). Ordinate, intensity; abscissa, *m/e*.

The presence of a significant odd mass peak at *m/e* 561 ($M^+ - 15$) and the equivalent chain length information supported a chemical of 41 carbon atoms. Three significant even mass peaks at *m/e* 224, 196, and 168 in the mass spectra (Figures 2–4) indicated that the branch points were positioned in the molecule so that secondary ion fragments with a long straight-chain tail could be formed. The presence of three predominant odd mass peaks at *m/e* 379, 407, and 435 confirmed three such methyl positions at the 15th, 13th and 11th carbon atoms. The three secondary ion fragments with the straight-chain tail could not be formed from one compound, thus the three even mass peaks at *m/e* 224, 196, and 168 must be from three different molecules (McCarthy et al., 1968). Although the mass peaks at *m/e* 168/169 and 196/197 could result from primary ion fragments (C_xH_{2x}/C_xH_{2x+1}) of different parent molecules, their intensities would be very small relative to the intensity of the secondary ion fragment with a hydrogen transferred (Nelson et al., 1972).

The presence of the predominant odd mass peaks at *m/e* 295, 267, and 239 indicated that each of these secondary ion fragments contained another methyl branch because an even mass peak of a secondary ion fragment with a straight-

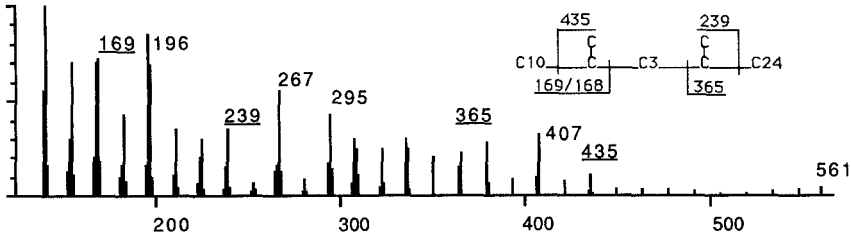


FIG. 4. Mass spectrum of a scan at 14 scans after the most intense scan with a mass range from 140 to 561: 11,15-dimethylnonatriacontane (most plausible structure). Ordinate, intensity: abscissa, *m/e*.

chain tail would be significant compared to the corresponding odd mass peak (McCarthy et al., 1968; Nelson et al., 1972). Therefore, these secondary ion fragments must have a methyl-branched tail. This agreed with the conclusion made from equivalent chain length determinations that two methyl branches were present. These three methyl positions were hypothesized to be at the 19th, 17th, and 15th carbon atoms. This was supported by the presence of the mass peaks at *m/e* 309 and/or 308, 337 and/or 336, and 365 since the molecular weight was 576. In addition, these three predominant odd mass peaks at *m/e* 259, 267, and 239 must come from three different molecules.

Generally, the internally multiple methyl-branched alkanes in the insects have isoprenoid spacing of the branch points (Nelson and Sukkestad, 1975; Nelson, 1978). Therefore, compounds in P-39B were postulated as three dimethylnonatriacontanes: 15,19-, 13,17-, and 11,15-dimethylnonatriacontane. Any one scan of the mass spectrum could not fully explain the existence of all three dimethylnonatriacontanes, but a survey of all the mass spectra scans revealed that the three dimethylnonatriacontanes existed. Based on the intensities of the specific mass peaks at *m/e* 196 and 407, and their appearance in almost all scans of P-39B, it seems that 13,17-dimethylnonatriacontane was the most abundant.

The characteristic fragments for the synthetic compounds are:

11,15-Dimethylnonatriacontane, EI mass spectrum at 70 eV [*m/e* (relative abundance)], 561[(0.8) M-15], 435[(5.3) $CH(CH_3)$-C_3H_6-$CH(CH_3)$-$C_{24}H_{49}$], 365[(4.2) $CH(CH_3)$-$C_{24}H_{49}$], 239[(7.9) $C_{10}H_{21}$-$(CH_3)CH$-C_3H_6-$(CH_3)CH$], 169[(5.2) $C_{10}H_{21}$-$(CH_3)CH$], 168[(9.6) $C_{10}H_{20}$-$(CH_3)CH$], 57[(base peak) C_4H_9].

13,17-Dimethylnonatriacontane, EI mass spectrum at 70 eV [*m/e* (relative abundance)], 561[(0.9) M-15], 407[(3.9) $CH(CH_3)$-C_3H_6-$CH(CH_3)$-$C_{22}H_{45}$], 337[(2.7) $CH(CH_3)$-$C_{22}H_{45}$], 336[(2.5) $CH(CH_3)$-$C_{22}H_{44}$], 267[(7.9) $C_{12}H_{25}$-$(CH_3)CH$-C_3H_6-$(CH_3)CH$], 197[(4.8) $C_{12}H_{25}$-$(CH_3)CH$], 196[(8.1) $C_{12}H_{24}$-$(CH_3)CH$], 57[(base peak) C_4H_9].

15,19-Dimethylnonatriacontane, EI mass spectrum at 70 eV [*m/e* (relative abundance)], 561[(0.9) M-15], 379[(4.2) $CH(CH_3)$-C_3H_6-$CH(CH_3)$-$C_{20}H_{41}$], 309[(3.4) $CH(CH_3)$-$C_{20}H_{41}$], 308[(3.8) $CH(CH_3)$-$C_{20}H_{40}$], 295[(8.7) $C_{14}H_{29}$-$(CH_3)CH$-C_3H_6-$(CH_3)CH$], 225[(4.0) $C_{14}H_{29}$-$(CH_3)CH$], 224[(6.1) $C_{14}H_{28}$-$(CH_3)CH$], 57[(base peak) C_4H_9].

Turning rates of *T. nubilale* females in areas treated with MSE, 13,17-DMC39, 15,19-DMC39, and 11,15-DMC39 were similar, and all but 11,15-DMC39 were significantly different from those in hexane treated areas (Table 1). Relative retention times in 13,17-DMC39-treated areas and in MSE-treated areas were the same and both retained the wasps significantly longer than the hexane control. Relative retention times in 11,15-DMC39- and 15,19-DMC39-treated areas were not significantly different from those in hexane-treated areas and were significantly lower than those in MSE- and 13,17-DMC39-treated

TABLE 1. MEAN TURNING RATE AND MEAN TIME SPENT BY *T. nubilale* IN AREAS TREATED WITH MSE AND SYNTHETIC HYDROCARBONS[a]

	MSE[b]	13,17-DMC39[b]	15,19-DMC39	11,15-DMC39	Hexane
Turning rate (turns/min)	23.3a[c]	24.5a	21.2a	19.3ab	14.2b
Time (%)	65.7a	70.5a	45.1b	43.4b	43.1b

[a]Results of nine replications, one female per replication.
[b]MSE = moth scale extract; DMC39 = dimethylnonatriacontane.
[c]Row means followed by the same letter are not significantly different by the multiple-range test of Duncan (1955) ($\alpha = 0.01$).

areas. Although 11,15-DMC39, 13,17-DMC39, and 15,19-DMC39 had klinokinetic effects on *T. nubilale* behavior similar to MSE, 11,15-DMC39 and 15,19-DMC39 failed to retain *T. nubilale* wasps in the treated areas. Therefore, 13,17-dimethylnonatriacontane is considered to be the most influential kairomone present.

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VARIABILITY IN PHEROMONE COMPOSITION AND PERIODICITY OF PHEROMONE TITER IN POTATO TUBERWORM MOTH, *Phthorimaea operculella* (LEPIDOPTERA: GELECHIIDAE)

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Abstract—The ratios and quantities of the pheromone components, (*E*,*Z*)-4,7-tridecadien-1-yl acetate (diene) and (*E*,*Z*,*Z*)-4,7,10-tridecatrien-1-yl acetate (triene), in the glands of individual female potato tuberworm moths (*Phthorimaea operculella*) originating from the United States (California) and Japan (Nagoya) were analyzed by gas chromatography. Quantities of gland-extracted pheromone components of Nagoya females fluctuated in a periodic fashion during the photoperiod. Maximal titers coincided with the onset of scotophase (and calling), then gradually declined to minimal levels soon after lights-on. The average daily pheromone quantities decreased significantly as females aged. Both populations exhibited considerable variation in the ratio of the two components. The proportions of triene in the blend ranged from 27% to 88% (triene $\bar{X}$ = 56 ± 13% SD; CV = 23%) for California females and from 16% to 71% (42 ± 13%; CV = 31%) for Nagoya females. Nagoya females also stored significantly higher amounts of pheromone in their glands (8.6 ± 3.9 ng) than did California females (2.7 ± 1.4). The differences between the populations, while substantial, would probably not be sufficient to impart a barrier to panmixis, given the wide range of component ratios favored by the males.

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Key Words—Sex pheromone, individual variation, pheromone titer, periodicity, (*E*,*Z*)-4,7-tridecadien-1-yl acetate, (*E*,*Z*,*Z*)-4,7,10-tridecatrien-1-yl acetate, Lepidoptera, Gelechiidae, potato tuberworm moth, *Phthorimaea operculella*.

INTRODUCTION

The female-emitted sex attractant pheromones of most moth species examined to date consist of two or more components. However, among the moths, the degree of specificity associated with production of pheromone blends by females and male response to these mixtures can vary markedly. For some species such as *Argyrotaenia velutinana*, *Grapholita molesta*, or *Trichoplusia ni*, a relatively precise blend of compounds is required for mate-finding by the males, and the attendant variation in the ratio of pheromone components produced by individual females is minimal (cf. Miller and Roelofs, 1980; Baker et al., 1981; Bjostad et al., 1984; Linn et al., 1986). In other species, exemplified by *Pectinophora gossypiella*, the females produce and emit a narrow range of ratios (Collins and Cardé, 1985; Haynes et al., 1984), whereas the males respond to a comparatively wider array of blend ratios (Flint et al., 1979; Linn and Roelofs, 1985). Species subscribing to a third condition, however, appear to utilize a more broadly tuned signal, with females producing, and males responding to an extensive range of certain or all components of the blend (Löfstedt et al., 1985; Attygale et al., 1986; Guerin et al., 1986; Barrer et al., 1987).

The sex pheromone of the potato tuberworm moth, *Phthorimaea operculella*, is a blend of (*E*,*Z*)-4,7-tridecadien-1-yl acetate (diene) (Roelofs et al., 1975; Persoons et al., 1976) and (*E*,*Z*,*Z*)-4,7,10-tridecatrien-1-yl acetate (triene) (Persoons et al., 1976). As determined from pooled samples of female abdominal tips or whole female extracts, the ratio of these two compounds is approximately equal (Persoons et al., 1976; Toth et al., 1984).

Field trapping studies in several countries and using a wide range of pheromone blends have shown that male *P. operculella* are lured almost equally well to a broad spectrum of ratios of diene to triene of 1 : 9 to 9 : 1 (Persoons et al., 1976; Voerman and Rothschild, 1978; Raman, 1984; Ono and Orita, 1986). In these tests, the blends generally captured more males than did the individual components, but in certain trials, the diene (Raman, 1984) or the triene (Voerman and Rothschild, 1978; Ono and Orita, 1986) alone captured as well as the mixtures. Given the broad range of blends suitable for attraction, a less precise regulation of pheromone component ratios in females of this species might be expected.

To document the variation among individual females in pheromone component ratios, we analyzed the gland content of females from the United States and Japan and found that there is indeed substantial variation in the quality and

quantity of the blends produced. In addition, we also describe how titers of both components vary according to the time of day and age of the females.

METHODS AND MATERIALS

Insects. The U.S. strain of *P. operculella* originated from laboratory stock supplied from Albany, California, and the Japanese colony was derived from insects collected from potato fields in Nagoya. Larvae were reared on potato tubers in plastic containers at 26±2°C and 70–80% relative humidity on a 14 : 10 light–dark photoperiod regime. Pupae were held individually in test tubes, and adult emergence was monitored daily 30 min prior to lights-off. Adults were maintained under the same conditions as larvae with access to water.

Pheromone Extraction and Quantification. Glandular pheromone content of individual females was analyzed by GLC. Virgin females were anesthetized by N_2 or CO_2 gas, and the abdominal tips including the pheromone gland (Adeeson et al., 1969) were excised. The glands were extracted for 1 hr in conical glass tubes containing 50 μl of either CS_2 (California strain) or hexane (Nagoya); 5 ng of (*E*)-11-tetradecenyl acetate or tetradecyl acetate was present as an internal standard in California and Nagoya samples, respectively. Preliminary tests showed that immersion of the glands ($N = 10$) for 1 hr in hexane or CS_2 extracted, respectively, 96 ± 5% ($\bar{X}$ ± SD) and 95 ± 7% of the pheromone present. Samples were stored in vials with Teflon-lined caps at −10°C until analysis.

Prior to analysis, solvent was evaporated from each sample using a gentle stream of N_2 gas until ca. 1 μl of solution remained. This method of sample concentration caused a reduction in the absolute amounts of each component but did not alter their ratio; following concentration and GLC analysis, a 1 : 1 diene–triene solution (5 ng/component in 50 μl hexane) yielded 48.9 ± 2.2% diene and 51.1 ± 1.9% triene (based on 10 samples analyzed on an SP-2340 capillary column).

Analyses were performed on Varian 3700 (California strain) or Shimadzu GC-9A (Nagoya) GLCs, both equipped with flame ionization detectors and splitless injectors. Chromatographs were fitted with SP-2340 fused silica capillary columns (30 m × 0.25 mm ID; 0.25- or 0.20-μm film thickness; Supelco, Inc.); nitrogen carrier gas flow was 2–3 ml/min. For the Varian chromatograph, the column temperature program was 120°C for 4 min, then a 5°C/min increase to a final temperature of 160°C for 10 min. The Shimadzu initial column temperature was 80°C, then programmed to 150°C at 20°C/min with a 10 min final hold. Both temperature profiles provided baseline resolution of the pheromone components and internal standards. Peaks were characterized by retention times, and peak areas were quantified by a Hewlett-Packard 3390A

electronic integrator. The limit of quantification for both systems was ca. 0.1 ng.

Pheromone Titer and Composition Analyses. To describe daily fluctuations in titer, glands of Nagoya females (ca. 30 generations in culture) were excised at 1600 hr (lights-off) of day 1 (one day postemergence) and every 4 hr thereafter for the ensuing four days. In addition, the titer of females 30 min following emergence was determined. Twelve to fifteen insects were analyzed for each time period.

Variation in pheromone composition was determined by analyzing the glands of individual California and Nagoya females on the second day following emergence (from 2 hr before until the onset of scotophase when titers attained maximal levels). At the time this study was conducted, Nagoya females had been in laboratory culture for five generations, whereas California females had been reared for >30 generations.

RESULTS

Daily and Age-Related Changes in Pheromone Titer. As shown in Figure 1, the titers of individual and combined components varied in a regular pattern throughout the diel period. Titers attained maximal levels at lights-off, corresponding to the beginning of the female calling period (Ono, 1977). Pheromone levels then gradually declined during scotophase to their low point centered on the onset of photophase; there was always some pheromone stored, even during periods of minimal titer. Immediately following emergence (<30 min), females also had appreciable levels of pheromone in their glands (total 4.8 ± 1.0 ng; triene 4.0 ± 0.9; diene 0.8 ± 0.6).

A stepwise multiple linear regression analysis (SPSS, 1975) was used to determine whether total pheromone titer varied in a periodic fashion. A 24-hr sine function [sine f(time)] with the 90° peak synchronized to 1600 hr (lights-off) was generated from the sample time variable; the sine function was used in the regression analysis with the sample time variable itself. The analysis revealed that the data conformed very well to this model [sine f(time): slope $\pm$ SEM $= 1.43 \pm 0.14$; $F = 103.6$; $P < 0.001$], confirming that pheromone titer cycled with a period of 24 hr.

Daily means were calculated by combining all titer determinations for a particular day ($N = 82$–88). Figure 2 shows that as females aged, the levels of the diene, triene, and total pheromone declined in a linear fashion so that by day 4 these amounts were roughly halved compared to day 1.

Individual Variation in Pheromone Ratios. Considerable variability in the ratio of the two components was apparent in the previous measurements at different daily times and adult ages (Figure 1). The pheromone composition of individual females ranged from no detectable triene (1 individual) to exclusively triene ($N = 42$).

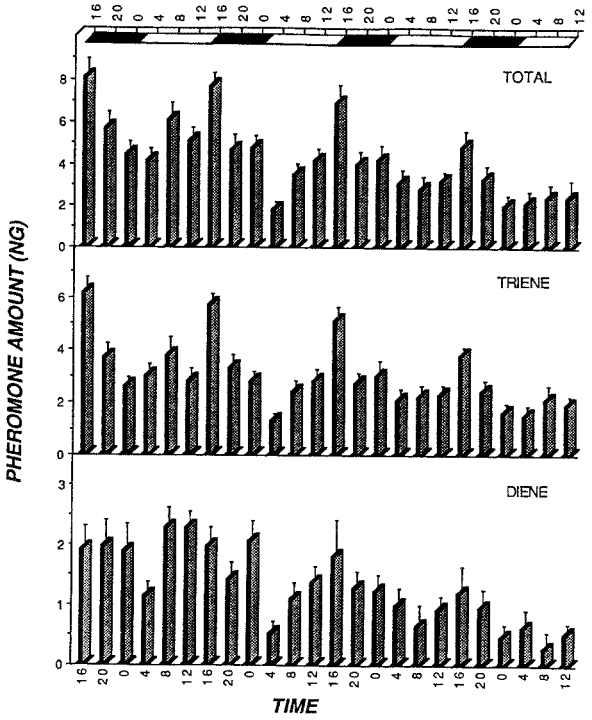

FIG. 1. Daily fluctuations in pheromone titer of 1- to 4-day-old Nagoya females whose glands were extracted at various times during the photoperiod. Each point represents the mean and associated standard error of 12–15 gland extracts. Shaded horizontal bars indicate scotophase.

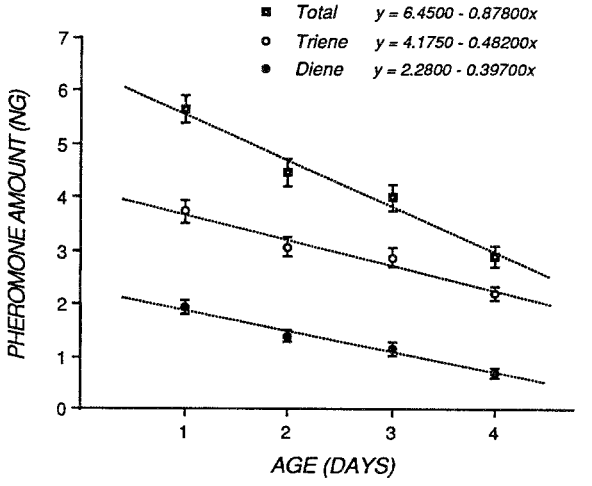

FIG. 2. Pheromone titers of Nagoya females as a function of age; each point represents the average of all titer determinations for a particular day (N = 82, 82, 88, and 84 for days 1, 2, 3, and 4, respectively). Vertical bars denote standard errors of the means.

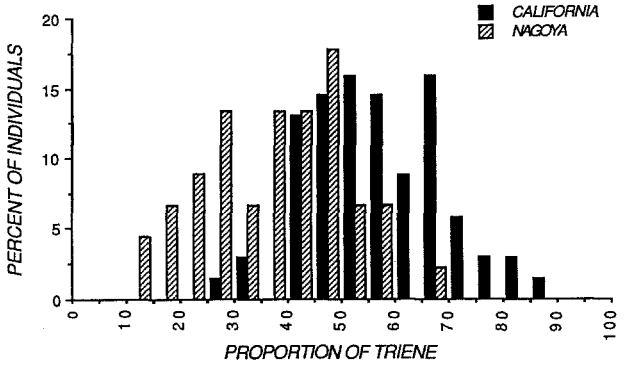

FIG. 3. Frequency distributions of the proportion of triene [triene/(diene + triene)] × 100 in the pheromone of California (N = 69) and Nagoya (N = 44) females. Glands of day 2 females were excised from 2 hr before until the onset of scotophase for both populations.

Individual variation in the composition of pheromone derived from California and Nagoya females extracted 2 to 0 hr before scotophase was also substantial. Figure 3 shows that the frequency distributions of the proportion of triene in the pheromone blends were broad for both populations. The range of triene proportions for the Nagoya females was 16.3–71.3% (equivalent to a diene–triene ratio of 0.4 : 1–5.2 : 1, or a 12.9-fold difference). The range of values was comparable for the California population: 28.2–88.2% (0.2 : 1–2.6 : 1, a 12.8-fold difference). For both populations, the mean ratio of diene to triene was approximately equal (Table 1); in the Nagoya population the diene tended

TABLE 1. PROPORTIONS AND AMOUNTS OF PHEROMONE COMPONENTS PRESENT IN GLANDS OF NAGOYA AND CALIFORNIA *P. operculella* FEMALES[a]

	Nagoya				California			
	Proportion		Amount (ng)		Proportion		Amount (ng)	
Compound	$\bar{X}$ (SD)	CV	$\bar{X}$ (SD)	CV	$\bar{X}$ (SD)	CV	$\bar{X}$ (SD)	CV
Diene	58.1(12.9)	22.2	4.8(2.1)	43.7	43.8(13.0)	29.6	1.2(0.7)	58.3
Triene	41.9(12.9)	30.8	3.8(2.3)	60.5	56.2(13.0)	23.1	1.5(0.8)	53.3
Total			8.6(3.9)	45.3			2.7(1.4)	51.9

[a] Derived from capillary GLC analyses of 44 Nagoya and 69 California individual female glands excised 0–2 hr before the onset of scotophase, two days after emergence. CV: coefficient of variation.

to be present in higher amounts [ratio 1.7 : 1 (1.1 SD); CV = 62.9)], whereas, on average, the triene was the more prevalent component in California females [0.9 : 1 (0.5); CV = 52.3]. The mean proportion of triene was ca. 15% higher in California females.

Glands of Nagoya strain females contained substantially more total pheromone, 8.6 ± 3.9 ng ($\bar{X}$ ± SD) than did those of their California counterparts (2.7 ± 1.4). Amounts of total pheromone extracted from Nagoya females ranged from 2.9 to 20.5 ng versus 0.4 to 6.3 ng for California females. Amounts of the individual and combined components also exhibited a high degree of variability in both strains, as is evident from the large associated coefficients of variation (Table 1).

DISCUSSION

The glandular titers of both *P. operculella* pheromone components fluctuated in a periodic fashion during the photoperiod. Peak amounts were present at the onset of scotophase; titers then declined during the dark period, attaining minimal levels soon after lights-on before beginning again to rise. Investigations on several other moth species have also revealed the existence of daily rhythms of pheromone production (e.g., Webster and Cardé, 1982; Raina et al., 1986; Snir et al., 1986; Delisle and McNeil, 1987; Dunkelblum et al., 1987a). Although we did not establish whether the cycling of pheromone titers in *P. operculella* has a circadian basis, circadian control of pheromone titer periodicity has been demonstrated in at least one moth, *Pseudaletia unipuncta* (Delisle and McNeil, 1987). Similar investigations will probably reveal that this is true for many moths.

The reduction in pheromone titer during the course of the scotophase may be attributable to emission of pheromone during calling and the resultant depletion of stored pheromone which is not offset by biosynthesis. Pheromone release in *P. operculella* commences at lights-off and ends at lights-on (Ono, 1977). An analogous pattern, characterized by the peak in titer preceding the peak female calling period, has also been documented for *Platynota stultana* (Webster and Cardé, 1982) and *Conogethes punctiferalis* (Konno, 1986). This type of pattern contrasts with the more widespread situation found in moths wherein peak pheromone titers either are essentially synchronous with maximal calling periods (e.g., Pope et al., 1982; Raina et al., 1986; Delisle and McNeil, 1987) or show little variation over the diel cycle (Sower et al., 1972; Schal et al., 1987).

Both populations exhibited substantial variation in the blend of components produced by the females. Moreover, the amounts of the individual components stored in the glands also were highly variable. The pooled quantities of com-

ponents extracted from both strains approximated a 1:1 ratio, similar to the ratios reported for pooled extracts of pheromone from other *P. operculella* populations (Persoons et al., 1976; Toth et al., 1984). In terms of amounts, Nagoya females produced 4.8 and 3.8 ng of the diene and triene, values similar to the 3.5 ng diene and 3.7 ng triene reported by Toth et al. (1984) for an Australian strain. The California strain produced substantially less pheromone (1.2 ng diene and 1.5 ng triene). Whether the lower titers reflect geographic variation or are the result of laboratory inbreeding depression is not clear. However, in this study, no significant differences were found in amounts of total pheromone from Nagoya females of comparable age that had been in laboratory culture for five generations (8.6 $\pm$ 3.9 ng; $N = 44$) versus ca. 30 generations (data from periodicity study: 7.7 $\pm$ 1.7 ng; $N = 15$).

Whether the ratio of the components in the glands of individual *P. operculella* females is indicative of the blend actually emitted remains to be determined. In other moths that utilize acetates in their pheromone, it has been found (at least in the 12- to 14-carbon-chain range) that the ratio of released components closely approximates their composition in the gland (Bjostad et al., 1984; Dunkelblum et al., 1987b), thus suggesting that the same situation may also apply to *P. operculella*.

The divergence in ratios of diene to triene between the California and Nagoya strains indicates substantial differentiation, but it seems unlikely in *P. operculella* that a ca. 15% difference in composition would impart any barrier to communication and mating, were the two strains to cooccur. Males are attracted to a broad spectrum of ratios (Persoons et al., 1976; Voerman and Rothschild, 1978; Raman, 1984; Ono and Orita, 1986), so that the difference in ratios produced by females from California and Nagoya is unlikely to be of behavioral significance. The Japanese population of *P. operculella* appears to have been introduced from Australia some 30 years ago (Koizumi and Ohshima, 1954), but it is not known if these populations are similar in either titer or component ratio.

Variation in pheromone ratio among individuals has been documented in several moth species. Miller and Roelofs (1980) showed that the mean proportion of (*E*)- and (*Z*)-11-tetradecenyl acetates extracted from the pheromone gland of wild *Argyrotaenia velutinana* females was 9.1 $\pm$ 1.8% SD, with a CV of 9.7%. (The CV is a useful measure for comparing relative variation among populations that differ in the magnitude of their means). Similarly, the mean ratio of (*Z*,*E*)- to (*Z*,*Z*)-7,11-hexadecadienyl acetate extracted from laboratory strain female *Pectinophora gossypiella* was 44.2 $\pm$ 2.3% SD, with a CV of 5.3% (Collins and Cardé, 1985).

In these species, the variation in male response appears moderately well linked to the limited variability in signal production. However, in *A. velutinana*, male attraction to blends about the natural ratio of (*Z*)- to (*E*)-11-tetra-

decenyl acetates is narrower (Baker et al., 1981; Linn and Roelofs, 1983) than *P. gossypiella* attraction to variants about its natural ratio (Flint et al., 1979; Linn and Roelofs, 1985). Such asymmetry in variability between female production and male response could reflect coding for differing strategies for signal selectivity in males and females. In general, males might be expected to be less narrowly tuned in response than females in signal production; this strategy could yield males responsive to all naturally available female ratios.

In *Agrotis segetum* (Löfstedt et al., 1985), variation in the ratio of the three pheromone components is substantial: 14.8 ± 127% CV for (*Z*)-5-decenyl acetate, 55.6 ± 32% CV for (*Z*)-7-dodecenyl acetate, and 29.6 ± 59% CV for (*Z*)-9-tetradecenyl acetate. Male attraction to dispensers loaded with a wide spectrum of blend ratios elicited only a 2.2-fold range of trap catch. In a similar study with *Ephestia cautella*, Barrer et al. (1987) also found a broad distribution of blend components; the individual composition of the two pheromone compounds, (*Z,E*)-9,12-tetradecenyl acetate and (*Z*)-9-tetradecenyl acetate, ranged from 63:27 to 97:3, with an overall mean of 88:12. These broadly tuned systems appear to be analogous to that of *P. operculella*.

Based on the available studies, it is possible to generalize about the relationship between the nature of the pheromone compounds used by moths and variation in blend quality. In general, blends composed of geometrical isomers (comprising either part or all of a particular pheromone) exhibit relatively low signal variance. Conversely, blends consisting of compounds that differ in the number or position of double bonds or that differ in chain length or functional moiety appear to have higher associated variability. The degree of variability associated with a given pheromone communication system may be a reflection of environmental and/or biosynthetic constraints. For example, among the latter systems, precise blend regulation may be unwarranted, because over the range of typical environmental temperatures and windspeeds, the ratio of the blend released can differ, not substantially, but by an amount above the range of variation found in tightly regulated blends (Cardé and Baker, 1984; but see Löfstedt et al., 1985). On the other hand, the variability may simply stem from inherent limitations in the ability of the moths to biosynthesize components in precisely regulated amounts. Roelofs and Bjostad (1984) have proposed that the diene and triene pheromone components of *P. operculella* are biosynthesized from different precursors, namely, linoleic and linolenic acid, respectively. Regulation of precise ratios of components derived from disparate precursors may not be as feasible as is the tight control of *Z* and *E* isomeric blends of acetates, because isomers of a particular acetate arise from the same saturated, long-chain fatty acyl precursor which itself is typically biosynthesized *de novo* (Roelofs and Wolf, 1988).

The extent to which congeners and perhaps unallied species employing similar communication systems cause a restriction of blend specificity will have

to await other evidence, such as would be provided by demonstration of reproductive character displacement in the chemical communication channel (Cardé, 1986). Species possessing a unique pheromone bouquet (as may be the case for the odd-carbon-chain components of *P. operculella*) would be under no selection pressure to maintain a narrowly tuned channel. Furthermore, evolution of a biosynthetic scheme yielding a precise ratio may only occur if there is an advantage, such as creation of an exclusive communication channel that avoids cross-attraction, to emitting a signal with a restricted variance (Löfstedt et al., 1985).

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COURTSHIP PHEROMONE PRODUCTION AND BODY SIZE AS CORRELATES OF LARVAL DIET IN MALES OF THE ARCTIID MOTH, *Utetheisa ornatrix*

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Abstract—Hydroxydanaidal, the corematal courtship pheromone of male *Utetheisa ornatrix*, shows pronounced quantitative variation in natural populations of the moth. Males that, as larvae, fed on seed-bearing rather than immature food plants (*Crotalaria spectabilis* or *C. mucronata*) produce higher levels of hydroxydanaidal. Such males also have higher systemic loads of pyrrolizidine alkaloid, the known metabolic precursor of hydroxydanaidal, which *Utetheisa* sequester from their larval diet and which is concentrated in the seeds of *Crotalaria*. Males raised on seed-bearing plants also achieve higher adult weight. In the context of sexual selection, therefore, female *Utetheisa* could, through assessment of male hydroxydanaidal levels, gauge both the alkaloid content and body weight of their suitors.

Key Words—Courtship pheromone, *Utetheisa ornatrix*, Lepidoptera, Arctiidae, sexual selection, hydroxydanaidal, pyrrolizidine alkaloid, *Crotalaria*.

INTRODUCTION

In this paper we investigate the natural variability in pheromone titer of a male moth and inquire into the possible cause of this variability. The moth in question, *Utetheisa ornatrix* (L.) (family Arctiidae), is one in which the chemistry

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of courtship is inextricably related to the chemistry of defense (Eisner and Meinwald, 1987).

Utetheisa, as larvae, feed on legumes of the genus *Crotalaria*, plants long known to be poisonous by virtue of the pyrrolizidine alkaloids they contain (Bull et al., 1968; Mattocks, 1986). *Utetheisa* sequester the ingested alkaloids and retain them systemically throughout development, deriving protection against predators as a result (Eisner, 1980; Eisner and Meinwald, 1987; Dussourd et al., 1988). Courtship in *Utetheisa* is initiated at dusk by release of a sex attractant from the female that lures the male. Upon reaching the female, the male flutters about her, while intermittently everting a pair of scent brushes (coremata) from his abdomen. The pheromone associated with the coremata, hydroxydanaidal, is derived by the male from the acquired pyrrolizidine alkaloid and is a key to the male's acceptance by the female. Males raised on a laboratory diet devoid of pyrrolizidine alkaloid fail to produce hydroxydanaidal and are relatively unsuccessful in courtship (Conner et al., 1981). These findings led to the suggestion that females, by favoring males of high hydroxydanaidal titer, could be sexually selecting for partners of high larval alkaloid-sequestering ability, a beneficial trait that could be heritable (Eisner, 1980; Conner et al., 1981). The subsequent discovery that males transfer a substantial fraction of the alkaloid to the female by seminal infusion, and that the female in turn bestows some of the "gift," together with alkaloid of her own, for protective purposes upon the egg, forced modification of the hypothesis. Hydroxydanaidal, it seemed, rather than serving strictly for appraisal of a larval trait, could be used by the female also for assessment of a male's potential alkaloidal offering (Dussourd et al., 1988). Quantitative data obtained with laboratory-reared *Utetheisa* provided some support for these notions. Males bore hydroxydanaidal at levels roughly proportional to their alkaloid load, and the amount of alkaloid they transferred to females at mating was itself proportional to this load (Dussourd, 1986). Missing from the scheme were values pertaining to *Utetheisa* in the field. No information was available on the natural variability of hydroxydanaidal in males and on whether such variability correlates with variation in larval alkaloid intake. The data we present here address these unknowns. Specifically we show that hydroxydanaidal levels vary over a broad range in nature, and that the titers of the pheromone are higher in males that had larval access to the alkaloid-rich parts (seeds) of their food plants. Moreover, hydroxydanaidal levels were found to correlate positively with adult male size.

In North Carolina and Florida where we study *Utetheisa*, the primary food plants of the moth are *Crotalaria spectabilis* and *C. mucronata*. The two plants differ qualitatively and quantitatively in pyrrolizidine alkaloid content. *C. spectabilis* produces primarily monocrotaline (Adams and Rogers, 1939); *C.*

mucronata mainly usaramine (Sawhney et al., 1967). The former produces substantially higher quantities of alkaloid (Williams and Molyneux, 1987). In both species the alkaloid is concentrated in the seeds and is present at substantially lower concentration in the leaves (Sharma et al., 1965; Johnson et al., 1985).

Our study involved analyzing adult males of two types, "wild" and "garden-raised," the former for corematal hydroxydanaidal content, the latter for both hydroxydanaidal content and systemic alkaloid load. Wild males were taken as adults from natural populations of the moth. Garden-raised males were reared outdoors on either mature (seed-bearing) or immature stands of both *C. spectabilis* and *C. mucronata*.

METHODS AND MATERIALS

Wild Males. Individuals were collected at five field sites, three in Moore County, North Carolina, and two in Highlands County, Florida. The sites consisted of disturbed wayside areas dominated by *C. spectabilis* or *C. mucronata*, or both. Individual males were captured by net and taken to the laboratory in plastic vials. Their coremata were then either excised at once (the elongate scent scales were removed with forceps, together with the underlying cuticle and glandular epithelium), or after live shipment to Ithaca, New York, and analyzed for hydroxydanaidal content.

Garden-Raised Males. These individuals were raised on eight stands of *Crotalaria*, planted outdoors in Durham, North Carolina. The stands were arranged in two replicate plots of four (within each plot the arrangement of stands was randomized). Within each plot the stands were as follows: one of 60 mature *C. spectabilis* plants with fully formed seedpods, another of 60 young *C. spectabilis* plants devoid of such pods, a third of 60 mature *C. mucronata* plants, and a fourth of 60 young *C. mucronata* plants. Each stand was "seeded" with 120 first-instar *Utetheisa* larvae, the offspring in each case of five females collected in Moore County, North Carolina. After approximately one month, surviving larvae, now in their fifth instar (ranging in number from 13 to 32 per stand) were retrieved from the stands and allowed to pupate in individual containers in an incubator (27°C; 16 : 8 light–dark photoperiod). Eclosing adults were maintained on 5% sucrose solution for seven days, then weighed and corematectomized. Coremata and bodies were analyzed for hydroxydanaidal and alkaloid content, respectively.

Chemical Analyses. The hydroxydanaidal contents of the coremata of wild males were determined by GC analysis using a Hewlett-Packard 5890 chromatograph equipped with a cool on-column injector, a 7673A autosampler, a flame ionization detector, and a 3392A recorder/integrator. Coremata were

weighed and placed in 1-dram vials to which methylene chloride (0.5 ml) and an internal standard (4 μg of benzophenone in 100 μl of methylene chloride) were added. Coremata were pulverized, further dispersed by sonication, and stirred for 24 hr. After centrifugation, the supernatant was removed; the remaining solids were extracted with two additional aliquots of 250 μl of methylene chloride. The supernatants were combined and their total volume reduced to ca. 200 μl. One microliter of this solution was injected into a wide-bore fused silica capillary column (0.53 mm × 25 m, 1 μM methylsilicone phase, Quadrex Corp.) using hydrogen (3.75 psi, 5 ml/min, 30 cm/sec) as the carrier gas. Column and injector were held at 35°C for 1 min, raised to 115°C at 25°C/min, raised to 145°C at 1.5°C/min, raised to 225°C at 25°C/min, and maintained at 225°C for 10 min. The retention times were 14.5 min and 21.0 min for hydroxydanaidal and benzophenone, respectively.

For the later analysis of the coremata of garden-raised males, the above extraction procedure was shortened to a 1-hr extraction in methylene chloride without reducing yields.

Body alkaloid analyses were carried out by a gradient HPLC technique using the pyrrolizidine alkaloid riddelliine as an internal standard. Samples were pulverized in a mixture of ethanol (2 ml) and mobile phase A (1 ml) containing a known quantity of riddelliine. Mobile phase A was prepared by dissolving 2.7 g of potassium dihydrogen phosphate, 2 ml of triethylamine, and 0.4 ml of trifluoroacetic acid in 4 liters of HPLC grade water. The pH was adjusted to 3.25 with phosphoric acid. The samples were stirred and sonicated for 24 hr. Each sample was then centrifuged and the supernatant drawn off. The residue was reextracted with 2 ml of mobile phase A for 24 hr, and the supernatants were combined. The combined extract was filtered into an autosampler vial.

HPLC separations were performed on a deactivated C-18 column (Supelco LC-18DB, 3 μM, 4.6 × 150 mm) at 35°C in an Eldex CH-150 column oven. An Eldex 9600 pump was used to deliver a mobile phase gradient [98% mobile phase A : 2% mobile phase B for 5 min (mobile phase B was prepared by adding 400 ml of acetonitrile and 10 ml of tetrahydrofuran to 1.6 liters of mobile phase A) changed linearly to 50% A : 50% B at 20 min]. The flow rate was programmed from 0.75 ml/min at injection with a linear increase to 1.25 ml/min at 25 min. Amounts of each alkaloid were determined based on relative responses observed from calibration standards of monocrotaline, monocrotaline *N*-oxide, usaramine, and usaramine *N*-oxide. All four alkaloids including riddelliine were resolved well by a UV detector at 205 nm (retention times: monocrotaline 7.0 min; monocrotaline *N*-oxide 13.5 min; usaramine 22.5 min; usaramine *N*-oxide 24.5 min; riddelliine 18.0 min).

RESULTS

Wild Males. The coremata of the field-collected males showed considerable variation in hydroxydanaidal content at all field sites (Table 1). Values did not differ significantly for the various sites (ANOVA; $P > 0.05$), justifying combination of the data into a single frequency diagram (Figure 1). The distribution is normal with a mean of 15.8 ± 8.2 μg hydroxydanaidal/male corematal pair.

Garden-Raised Males. Host plant age and species had a significant effect on corematal hydroxydanaidal levels (two-way ANOVA) (Figure 2). Males raised on mature, pod-bearing plants had higher hydroxydanaidal levels than males raised on podless plants ($P < 0.01$). Individuals raised on *C. spectabilis* had higher levels of hydroxydanaidal than individuals raised on *C. mucronata* ($P < 0.01$). No significant interactions were detected.

Significant differences were also apparent in the alkaloid content of males of the four categories (two-way ANOVA) (Figure 3). For both plant species, males raised on mature plants had higher alkaloid loads than individuals from podless plants ($P < 0.01$). Individuals raised on *C. spectabilis* had higher overall alkaloid levels than individuals raised on *C. mucronata* ($P < 0.01$). No significant interactions were detected. The particular alkaloid in the moths matched the type found in their food plant: moths raised on *C. spectabilis* contained monocrotaline, while those raised on *C. mucronata* contained usaramine. In all moths the alkaloid was present predominantly in the *N*-oxide form. The free base form accounted for less than 5% of the total alkaloid. The data are presented as sums of *N*-oxide and free base.

The data revealed a further correlate of larval diet. Adult male weight was found to be significantly greater for individuals raised on mature plants than for those raised on immature plants (79.4 ± 26 μg vs. 49.3 ± 1.8 μg; two-way

TABLE 1. HYDROXYDANAIDAL TITERS IN COREMATA OF MALE *Utetheisa ornatrix* FROM FIVE SITES

Site	Location	Host plant	*N*	Mean ± SD (μg)
1	Highlands County, Florida	*C. mucronata*	25	18.2 ± 7.5
2	Highlands County, Florida	*C. mucronata*	47	15.1 ± 7.7
3	Moore County, North Carolina	both species	13	14.4 ± 11.9
4	Moore County, North Carolina	*C. spectabilis*	11	12.5 ± 5.1
5	Moore County, North Carolina	both species	17	17.7 ± 8.6

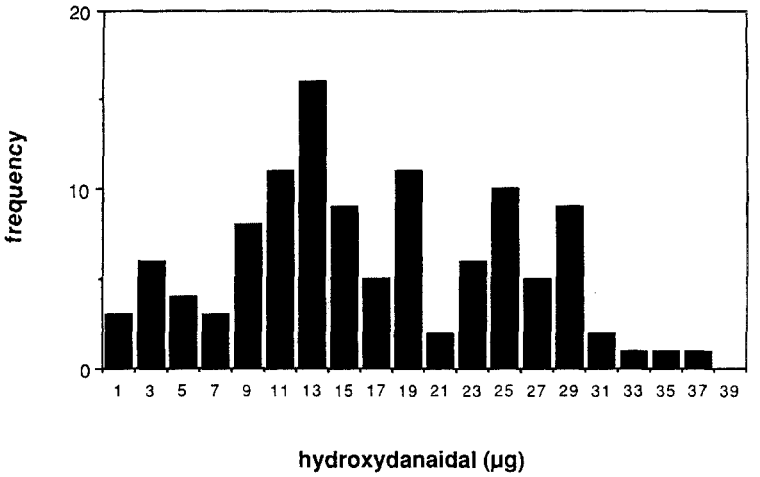

FIG. 1. Variation in hydroxydanaidal content of coremata of field-collected male *Utetheisa*. Combined data from five field sites.

ANOVA; $P < 0.01$). There was no significant difference in the weights of males raised on the two plant species ($P > 0.05$).

Hydroxydanaidal levels correlated significantly (simple linear regression; $P < 0.05$) with both the total alkaloid content and weight of males, but each

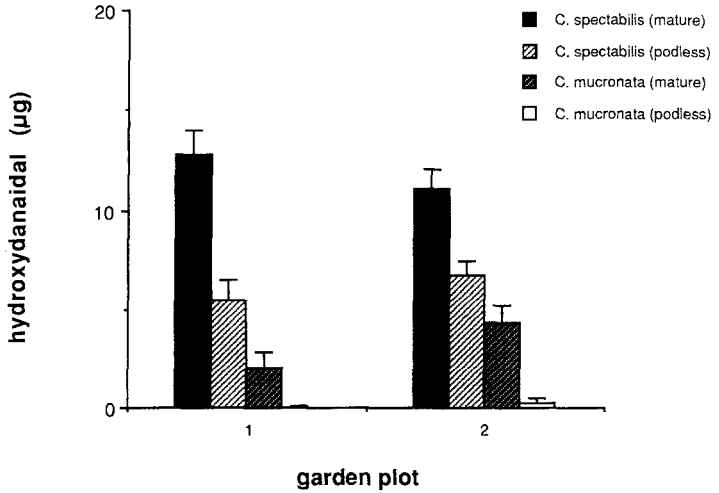

FIG. 2. Hydroxydanaidal titer of coremata of male *Utetheisa* raised on gardens of four types, with replication. Bars indicate standard errors.

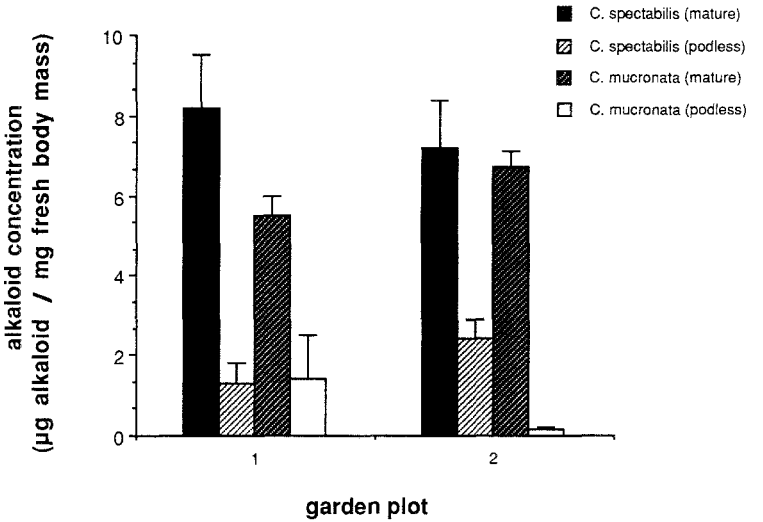

FIG. 3. Pyrrolizidine alkaloid concentration of male *Utetheisa* raised on gardens of four types, with replication. Bars indicate standard errors.

variable accounted for less than 20% of the total hydroxydanaidal variance. The correlations improved when the data from the *C. spectabilis* and *C. mucronata* stands were analyzed separately. For the *C. spectabilis* stands, alkaloid content and weight accounted for 36.1% and 59.0% of the variance, respectively (Figure 4). The corresponding values for the *C. mucronata* plots were 25.1% and 39.7% (Figure 4).

DISCUSSION

It seems established that corematal hydroxydanaidal levels in male *Utetheisa* vary over a broad range in natural populations and that the levels of the pheromone are affected by larval diet. The finding that hydroxydanaidal levels were highest in males with larval access to pod-bearing plants, as opposed to podless plants, makes sense, given that hydroxydanaidal is derived from ingested alkaloid and that the alkaloid is maximally concentrated in the seeds of *Crotalaria*. We know from field observation that *Utetheisa* feed avidly on the seeds of their food plants, and we noted them to do so on our experimental stands. As expected, access to pods also resulted in higher systemic alkaloid loads. In the context of courtship, therefore, by gauging a male's hydroxydanaidal titer, a female could be appraising both her suitor's dietary history and alkaloid load, assessments by which she could exercise mate choice. While it remains to be proved that *Utetheisa* females discriminate behaviorally between incremental

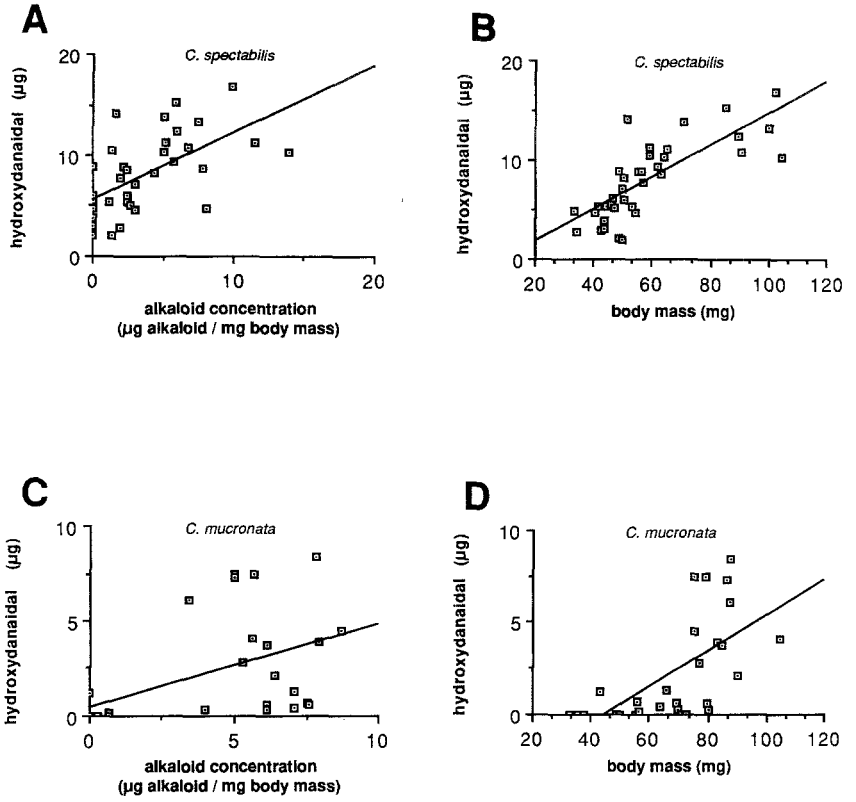

FIG. 4. (A) Correlation between total alkaloid load in male *Utetheisa* raised on *C. spectabilis* and the hydroxydanaidal content of their coremata. (B) Correlation between body mass of males (same individuals as A) and the hydroxydanaidal content of their coremata. (C) Correlation between total alkaloid load in male *Utetheisa* raised on *C. mucronata* and hydroxydanaidal content of their coremata. (D) Relationship between body mass of males (same individuals as C) and the hydroxydanaidal content of their coremata.

levels of hydroxydanaidal and that they select suitors on that basis, it is known that they can differentiate between presence and absence of the pheromone and that they favor males that have the compound (Conner et al., 1981). Electrophysiological data on antennal chemoreceptors do predict that the female should be able to discriminate between even slight concentration differences of hydroxydanaidal (Grant et al., 1989).

The correlation between hydroxydanaidal level and alkaloid load (Figures 4A and C) is far from tight. Nonetheless, within limits, hydroxydanaidal could serve as a predictor of male alkaloid content, and this could well be one role of the pheromone.

Additional complexity is introduced by the finding that hydroxydanaidal titers also correlate with male size. Not surprisingly, males with larval access to the more nutritious seed-bearing plants achieved greater adult weight. Hydroxydanaidal could thus provide the female also with a measure of male size and, indirectly, spermatophore size (do males transmit nutrients in addition to alkaloid with their spermatophores and is the amount transferred a function of male size?). The spermatophores of *Utetheisa* are large, averaging 7.5% of male body weight (Dussourd, 1986) and their size correlates with male body weight in laboratory-raised individuals (David Dussourd, personal communication). A similar relationship between male courtship pheromone levels and male size has been reported for the tobacco moth, *Ephestia elutella* (Phelan and Baker, 1986).

In our view, the finding that hydroxydanaidal levels are widely variable in nature need not indicate solely that the moths had larval access to plants of different maturational age. It also could signify that the moths had a history of competing for seeds on mature plants and that they did so with varying success. Field observations support this notion. We often observed in natural stands of *C. mucronata*, when *Utetheisa* larvae were dense, and especially when another seed-eater, the larva of the pyralid moth *Etiella zinckenella* (Treit.), was also abundant, that virtually the entire seed set of the plants was eaten away. *Utetheisa* males with minimal levels of hydroxydanaidal, such as we now know to occur in nature, could thus represent the losers in such combined intra- and interspecific competition, rather than merely individuals that fed on immature plants.

The hydroxydanaidal values presented here are substantially higher than those we initially reported for *Utetheisa* (Conner et al., 1981). We attribute this to differences in the corematectomy techniques used. By the earlier method we snipped off and extracted the corematal scales only, while for the current extractions we excised the underlying glandular tissue as well.

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BIOLOGY OF PHEROMONE RELEASE BY MALE CARIBBEAN FRUIT FLIES, *Anastrepha suspensa* (DIPTERA: TEPHRITIDAE)[1]

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Abstract—Males of the Caribbean fruit fly, *Anastrepha suspensa* (Loew), typically form leks and attract females by releasing a multicomponent volatile pheromone. Previous reports have identified two nine-carbon alcohols, three lactones, a sesquiterpene, and a monoterpene in the volatiles. The present report is a study of the physiology of male pheromone release and of ecological and social interactions that influence pheromone release by laboratory-reared flies. Volatiles released by males were trapped on Tenax, eluted, separated, and quantitatively measured by gas chromatography. Experiments showed that the volatiles were primarily released from mouth and anus. Sealing the anal opening or the mouth with melted beeswax resulted in up to 40% or greater reduction in most components, and sealing both mouth and anus further reduced release of volatiles, but some volatiles are possibly still released directly from the cuticle. An anal pouch of everted tissue played a major role as a large evaporative surface for release of some of the volatile components. Male flies entrained to a 14 : 10 light-dark cycle showed a peak release of volatiles at 11-12 hr into the photophase, but smaller quantities of the same volatiles were released over a broad period during the daylight hours. Laboratory-reared males peaked in pheromone release at 7-10 days and production and release continued through 35 days of age. Single males released significantly more of all components measured than did groups of males. The reduction by aggregations of males may be related to lekking behavior in this fruit fly. The pheromone probably serves to attract females to a lek site, but additional parameters are likely to enter into the choice of male made by the arriving female.

Key Words—*Anastrepha suspensa*, Diptera, Tephritidae, fruit flies, pheromone, volatiles, lek, circadian rhythm, mating behavior.

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INTRODUCTION

Male Caribbean fruit flies, *Anastrepha suspensa*, attract females, at least in part, by release of a volatile pheromone, as shown in both laboratory and field experiments (Nation, 1972; Perdomo et al., 1975, 1976; Dodson, 1982; Burk, 1983; Webb et al., 1983). In field tests, but not in lab tests, males are also attracted to sticky traps baited with live males (Perdomo et al., 1976) and aggregations of males have been observed in the field (Perdomo, 1974; Burk, 1983). Several authors (Dodson, 1982; Burk, 1983; Sivinski, 1984) have considered the behavioral ecology of Caribbean fruit flies to include lekking behavior, a mating system in which males aggregate and attract females to a lek arena for mating (Alexander, 1975; Bradbury 1981). Lek behavior has been recognized in a number of insects (Spieth, 1970; Alexander, 1975; Willis and Birch, 1982), including some other tephritid fruit flies (Tychsen, 1978; Morgante et al., 1983; Prokopy and Hendrichs, 1979; Kuba et al., 1984; Dodson, 1986).

The volatile pheromonal components released by Caribbean fruit fly males have been identified and synthesized, and include (Z)-3-nonenol, (Z,Z)-3,6-nonadienol (Nation, 1983), two chiral lactones, anastrephin and epianastrephin (Battiste et al., 1983), and a nonchiral lactone, suspensolide (Chuman et al., 1988). In addition to these, unpublished data from this laboratory and a verbal report from another laboratory (J. Tumlinson, 1987, "Pheromone chemistry: Getting it right," Annual Meeting, Entomological Society of America, Boston, Massachusetts) have identified the presence of β-bisabolene and β-ocimine in the volatiles, but their behavioral roles are uncertain. While the two nine-carbon alcohols and the two gamma lactones have individual behavioral activity in bioassays with female flies (Nation, 1975, and unpublished data), the behavioral role of the macrolide is not well defined. Laboratory bioassays show that it has some behavioral activity upon females (unpublished data).

A general review of the behavioral and chemical ecology of the Caribbean fruit fly and related fruit flies, as well as preliminary details from collection of the volatiles of the male flies, has been reported (Nation, 1989). In that review, the then incompletely identified suspensolide was referred to as lactone B, bisabolene was identified only as a sesquiterpene, and ocimine as a monoterpene. This paper presents a more detailed report of volatile collections and of environmental and behavioral factors that influence the quantity and blend of pheromone volatiles released by male flies.

METHODS AND MATERIALS

Insects. A. suspensa were obtained from a laboratory colony of flies reared according to the procedures of Greany et al. (1976). The colony has been in continuous culture since about 1966. Adults were maintained on dry yeast

hydrolysate, dry sucrose, and water fed ad libitum. Adult flies were segregated by sex into cloth-covered aluminum-frame cages on the first day after emergence or earlier and held in separate incubators at 26–27°C with a 14 : 10 light–dark cycle.

Collection of Volatiles: Volatiles were collected from male flies as previously reported (Nation, 1989) by utilizing a water pump to draw air over flies in a cylindrical glass vessel. Males were placed into the collection device a few minutes before the experiment was to begin. A cylindrical glass vessel was used to hold males during the course of the collections (Figure 1A). Cylinder 1 was 38 mm ID and 235 mm long. A screen cap at the air inlet end retained the males, while an aluminum foil-covered rubber stopper with a glass tube passing through closed the other end and provided an exit tube for air and volatiles. Cylinder 1 was used to collect all data except data for Figure 4. Collection of Figure 4 data was made with cylinder 2, which measured 34 mm ID and 173 mm long. A screen at one end retained males and admitted air. The opposite end of the cylinder was drawn into a single exit tube 6 mm in diameter. Air flow over male flies in the cylinder was adjusted to 0.8–1 liter/min and was led from the exit tube in each cylinder into two traps in series, each containing 300 mg Tenax GC as the adsorbant (Cross, 1980; Golub and Weatherston, 1984). The column of adsorbent was about 3 cm long and was held in glass tubes (12 mm ID, 18–20 cm long) between plugs of silanized glass wool. After a predetermined time of collection, traps were removed and volatiles were eluted from the Tenax with pentane. Some collections were made with a prefilter of Tenax or charcoal at the air intake (ground glass joint) end of the apparatus (Figure 1B), but in practice the prefilter proved unnecessary most of the time and often it was not used. If contaminants showed on the chromatograms, the prefilter was reinstated (as for example, when the interior of the building was being repainted).

In preliminary tests designed to determine possible breakthrough of volatiles from the first trap, the two traps were eluted separately, each with 10 ml pentane, and subsequent GC analyses conducted on the eluates. After conditions to minimize breakthrough were established, the eluate from trap 2 was allowed to drip into trap 1 and washing of the two traps was continued until 10 ml effluent was collected from trap 1. In some collections from a single male for 1 hr, only one trap of 300 mg Tenax was used. Poropak and coconut charcoal (Fisher Scientific Co., activated coconut charcoal, 50–200 mesh) were also tested as trapping agents.

Preliminary experiments revealed that addition or omission of food and water from males for the usual collection period of 3 hr had no effect upon quantity or composition of volatiles collected. Thus, no food or water was placed in the collection apparatus with males if the experiment was to last only 3 hr. For longer experiments, dry sucrose, dry yeast hydrolysate, and water were placed in the apparatus in separate vial caps. Although yeast hydrolysate released

some volatiles, the quantity was minor, and fortunately none of them interfered with the measurement of the male produced volatile components.

Determination of Release Sites for Volatiles. Volatiles were collected from male flies with either the mouth or anus, or both sealed with melted beeswax. Control males had wax deposited on the thorax. Care was taken to heat the wax just to the melting point in order to avoid injury to flies by wax that was too hot. At the end of a collection period, males were examined by viewing under 10–30× magnification to see if the wax still sealed the mouth or anus. Generally, failure of the wax seal was not a problem, but if males were found that had broken the seal loose, the experiment was discarded.

Determination of Influence of Photoperiod, Age, and Male Density on Release of Volatiles. Collection of volatiles from a group of 25 males was begun at 6 hr into the photophase and the Tenax traps were changed hourly to determine the time and rhythm of volatiles release. The same 25 males were retained in the apparatus for the entire day of collection and they were provided with dry sucrose, dry yeast hydrolysate, and water.

To determine the influence of age of males upon the quantity and composition of volatiles released, collections were made from single males or from 25 males of known age in the collection apparatus for a period of 3 hr that included the 11th through the 13th hours of the photophase. Males taken at random from the same cohort of flies were used on successive days for the age study.

The influence of the number of males in the holding chamber upon volatiles released was determined with two cylinders (type 1) placed side by side on the table top. One apparatus contained a single male, while the second apparatus contained two, three, five, or 10 males. Additional experiments were also conducted in which one apparatus contained three or five males, while the other contained a larger group. All males were from the same cohort of flies and were taken at random from a large cage of males held in the incubator. Volatiles were collected for 3 hr during the 11th through the 13th hours of the photophase. Care was taken to adjust air flow equally in the two systems.

Chromatography and Quantitation of Volatiles. An internal standard containing decanol, tetradecane, and methyl tridecanoate was mixed with the eluted pheromone solution and the volume was reduced to about 0.5 ml by a gentle jet of nitrogen gas. The quantity of each of the three internal standards was adjusted from time to time to produce GC peak areas similar to the peak area of the component(s) to be estimated by that standard. For example, smaller quantities of standards were used in measuring collections of volatiles from a single male than were used in measuring collections from a group of males. Suitability of the three internal standards for quantitation of particular pheromonal components was determined from gas chromatographic analyses and measurement of peak areas produced from weighed samples of pure synthetic components and internal standards.

The mixture of volatile components and internal standards was separated by gas chromatography with a Varian 3700 or Varian 2400 gas chromatograph equipped with a flame ionization detector and interfaced with an HP 3390 A recorder–integrator. The chromatographs contained a 10 m × 0.5 mm ID wide-bore RSL 150 or RSL 160 Non-Pakd column (Alltech Associates) or a 30 m × 0.25 mm ID DB-1 (J&W Scientific, Inc.) capillary column. Injections into the wide-bore columns were on-column and splitless, and splitless into the capillary columns. Injections into the RSL columns were made at 105°C with immediate programming at 1°C/min to 170°C. Helium carrier gas flow was 3 ml/min with no additional makeup gas flow. The injector port was held at 165°C and detector at 185°C. Injections into the DB-1 column were made at 50°C, held for 5 min, then programmed at 2°C/min to 150°C, and held for 45 min. The injector port was held at 180°C and the detector at 250°C. Helium linear flow was 16.1 cm/sec, with makeup gas flow approximately 25 ml/min.

Data Analysis. Comparisons of the quantity of individual volatile components released by male flies were made with a paired Student's *t* test (Stat-Pak software). The 0.05 probability level was chosen for rejection of the null hypothesis that release rates of specified volatile components were equal in the various experimental situations. The relationship between levels of volatile components was also examined by linear regression.

RESULTS

Validation of Internal Standards as Quantitative Estimators of Pheromonal Volatiles. The accuracy and reproducibility of decanol, tetradecane, and methyl tridecanoate as quantitative estimators of the components in the volatiles from male flies are shown in Table 1. Decanol gave excellent estimation of a weighed sample of (Z)-3-nonenol (97.4 ± 3.7%, mean ± SEM), and it was chosen to

TABLE 1. EVALUATION OF INTERNAL STANDARDS FOR ESTIMATION OF PHEROMONAL COMPONENTS IN VOLATILES COLLECTED FROM MALE *A. suspensa*[a]

Synthetic pheromonal component	Internal standard (% of actual found)		
	Decanol	Tetradecane	Methyl tridecanoate
(Z)-3-Nonenol (N = 9)	97.4 ± 3.7	78.3 ± 2.9	90.1 ± 2.5
β-Bisabolene (N = 11)	137.4 ± 3.5	109.1 ± 2.5	127.1 ± 3.2
Epianastrephin (N = 11)	106.2 ± 3.4	82.9 ± 2.3	98.4 ± 3.1

[a] Synthetic pheromonal and internal standards were weighed and dissolved in pentane or isooctane, and chromatographed by GC. Mean percent (± SEM) of weighed quantity found.

estimate the two nine-carbon alcohols, (Z)-3-nonenol and (Z,Z)-3,6-nonadienol, which eluted together and just before decanol on the columns used. Tetradecane, a straight-chain hydrocarbon, slightly overestimated a weighed sample of bisabolene (109.1 ± 2.5%), but it was used as the internal standard for estimating bisabolene without correction. Methyl tridecanoate allowed 98.4 ± 3.1% estimation of a weighed sample of epianastrephin and was used to measure anastrephin, epianastrephin, and suspensolide. The best standard for estimating suspensolide and an evaluation of accuracy cannot be determined because very pure synthetic suspensolide is not available for gravimetric preparation of a standard. All pheromonal components collected (except β-ocimine) were calculated on the basis of a 1 : 1 detector response with the internal standard used. β-Ocimine was not routinely measured because it elutes too rapidly under the chromatographic conditions used for the other components. Some preliminary analyses of ocimine were reported earlier (Nation, 1989), indicating that β-ocimine is a very minor component in the volatiles, and its behavioral status is also uncertain.

Evaluation of Trapping Agent for Pheromonal Volatiles. Preliminary experimentation with the quantity of Tenax to use in the collecting traps, as well as experiments to see if breakthrough of the volatile components occurred, showed that two Tenax traps, each containing 300 mg Tenax GC, would trap all the volatiles from up to 50 males for several hours (Table 2). Some breakthrough of suspensolide and epianastrephin into trap 2 occurred when large collections were made from 50 males over a period of 6 hr. None of the other compounds showed breakthrough. In typical collections of volatiles from a few males for up to 3 hr, only trace amounts of suspensolide broke into trap 2. At

TABLE 2. RECOVERY OF VOLATILE COMPONENTS FROM *A. suspensa* MALES IN TWO TRAPS CONNECTED IN SERIES AND EACH CONTAINING 300 MG TENAX GC[a]

		Total Found (μg)[b]				
		N	S	B	A	E
Trial 1	Trap 1	44.6	72.9	165.7	37.6	158.0
	Trap 2	none	64.4	none	none	6.7
Trial 2	Trap 1	61.0	45.3	185.6	36.3	153.1
	Trap 2	none	67.4	none	none	13.7

[a] Air flow over males and into traps was provided by a water vacuum pump. The air flow rate was adjusted to 0.8–1 liters min. Volatiles were collected continuously from 50 males for 6 hr.
[b] N = (Z)-3-nonenol and (Z,Z)-3,6-nonadienol; S = suspensolide; B = β-bisabolene; A = anastrephin; E = epianastrephin.

the end of the collection period the two Tenax traps were disconnected and trap 2 was clamped above trap 1. Elution of the trapped volatiles was started by adding pentane at the top of trap 2. The eluate from trap 2 dripped into trap 1, and washing of both traps was continued until 10 ml eluate was collected from trap 1. About 5 ml of pentane was sufficient to elute all collected volatiles from the two 300-mg traps, but elution was routinely continued until 10 ml eluate had been collected to ensure quantitative collection. Periodic checks by gas chromatography of a few milliliters eluted after 10 ml was collected showed that elution from the two traps was complete.

Poropak was discarded as a trapping agent after preliminary use because it was difficult to get it as clean as Tenax. Charcoal was tried and discarded because it was more difficult to elute the polar compounds in the volatiles from charcoal traps. Ten milliliters of pentane, which was more than sufficient to quantitatively elute all compounds from the Tenax traps, eluted less than 50% of the adsorbed anastrephin and epianastrephin from two charcoal traps. Those compounds could be eluted with ether.

Quantitative Analysis of Collected Volatiles. A typical chromatographic record obtained from the RSL 160 megabore column is shown in Figure 1. Identities of the peaks in Figure 1 were based on chromatographic comparison with authentic synthetic standards for compounds previously identified from male Caribbean fruit flies and included (*Z*)-3-nonenol and (*Z*,*Z*)-3-6-nonadienol (coeluting), suspensolide, β-bisabolene, anastrephin, and epianastrephin. Since (*Z*)-3-nonenol and (*Z*,*Z*)-3,6-nonadienol eluted together as one peak in the chromatographic systems used, they are henceforth referred to in this report as nonenols. Measurements of components left on the collecting vessel walls showed that 6–8% of the anastrephin and epianastrephin was left on the vessel walls, while residues of other components were not detected.

The data in Table 3 indicate that the pheromonal volatiles were liberated from both mouth and anus. When either the mouth or anal opening, or both, were sealed with melted beeswax, the quantity of all pheromonal volatiles was reduced. There was from 35% to greater than 50% reduction in volatile components when the mouth was sealed. This would prevent the flies from releasing components from the mouth and would also prevent them from swallowing air that might be passed through the gut. Sealing of the anus resulted in nearly as great reductions as when the mouth was sealed. When both mouth and anus were sealed with wax, there was 62–76% reduction in volatiles released. Probably some or most of the pheromone collected with both mouth and anus sealed was already on the body surface.

The anal pouch (Figure 2) that males evert when releasing pheromone is shiny and has occasionally been mistaken for a droplet of liquid at the tip of the abdomen (Burk, 1981; and cited by Prokopy and Roitberg, 1984). The pouch is the everted lining of the posterior portion of the rectum. It undoubtedly con-

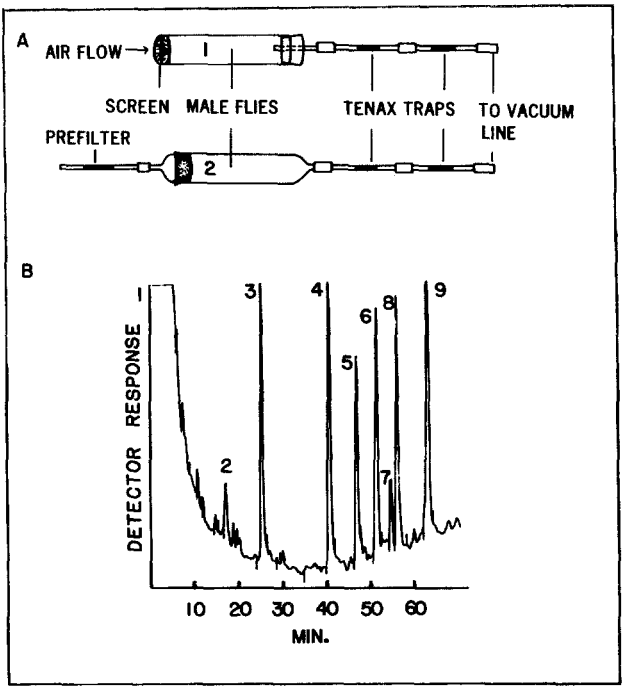

FIG. 1. (A) Diagram of the two types of collection devices used. (B) Gas chromatographic separation of volatiles from a single male *A. suspensa*. The record is from a 10 m RSL 160 Non-Pakd column, 0.5 mm ID. Peak identities are: 1, solvent; 2, combined quantities of (Z)-3-nonenol and (Z,Z)-3,6-nonadienol; 3, decanol (internal standard); 4, tetradecane (internal standard); 5, suspensolide; 6, β-bisabolene; 7, anastrephin; 8, epianastrephin; and 9, methyl tridecanoate (internal standard).

TABLE 3. PERCENT (MEAN ± SD, $N = 13$) REDUCTION IN PHEROMONAL COMPONENTS RELEASED BY MALE *A. suspensa* WHEN MOUTH (MS) OR ANAL OPENING (GS) WAS SEALED WITH BEESWAX, OR BOTH WERE SEALED (MS, GS; MEAN ± SD, $N = 2$)[a]

	Percent Reduction			
	N	S	B	A + E
MS	34.7 ± 8.30	54.1 ± 26.4	51.9 ± 16.2	59.6 ± 17.8
GS	22.2 ± 17.2	37.1 ± 17.5	38.9 ± 9.4	47.0 ± 17.6
MS, GS	75.8 ± 15.8	72.3 ± 17.9	62.1 ± 24.3	67.5 ± 14.4

[a]N = (Z)-nonenol and (Z,Z)-3,6-nonadienol; S = suspensolide; B = β-bisabolene; A + E = anastrephin plus epianastrephin.

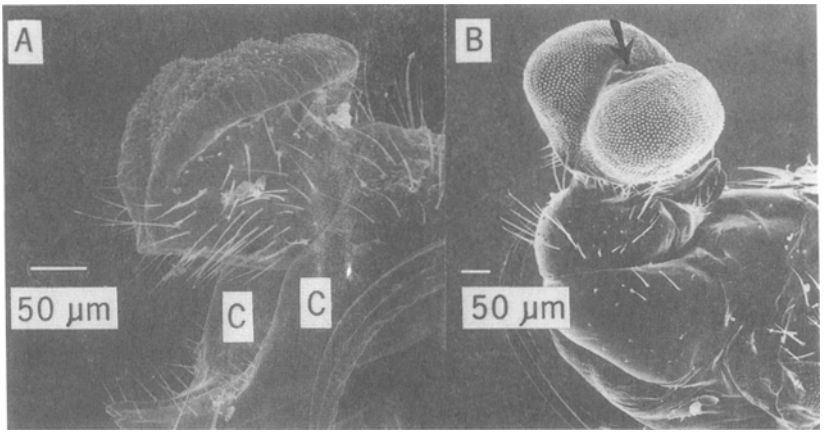

FIG. 2. A scanning electron micrograph of the anal pouch of male *A. suspensa*. (A) The pouch is only partially everted; (C) male clasper. (B) The pouch is fully everted. The arrow indicates the anal opening.

tains pheromone on its surface. Males repeatedly touch or dab the pouch to the substrate surface when behaviorally displaying (Nation, 1972). Males also repeatedly touch the labellum to the substrate surface, sometimes leaving tiny droplets that can be observed, and it seems very likely that they are depositing pheromone on the substrate by this behavior.

Influence of Photoperiod and Age upon Pheromone Release. A daily rhythm strongly influences quantity and composition of volatiles released (Figure 3). During the early morning hours of the photophase the quantity of all components released is very low, but later rises to reach a broad maximum extending from about 11 through 13 hr into the photophase. Release declines toward the end of the photophase. Males kept in the light, however, beyond the normal 14-hr photophase showed continued declining volatile release for the next 5–6 hr, beyond which collections were not continued (unpublished data). The total quantity of components and ratios of components in the volatiles changed hourly and in unpredictable ways, and neither quantity nor ratios were constant for any particular hour from day to day.

A pattern of changing release rate with a high degree of unpredictability was found also in the influence of age upon volatiles release (Figures 4 and 5). Although nonenols could be collected from 1- and 2-day-old males, the total complement of volatile components could not be detected in collections earlier than about 4 days of age and then only in extremely small quantities. The levels increased rapidly, however, and reached a maximum usually at about 7–9 days in the laboratory-reared strain of flies. Feral flies, which have a much longer

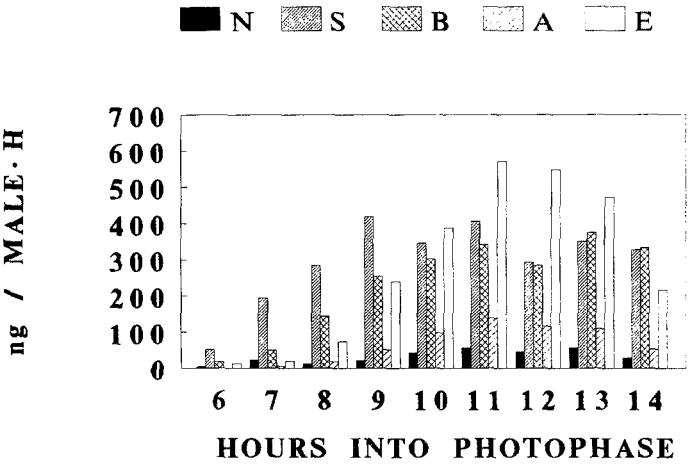

FIG. 3. Volatile components (ng/male/hr) collected from a group of 25 male *A. suspensa*, 12 days old, during successive hours in the photophase. Males were kept on a 14 : 10 light–dark cycle. N, S, B, A, and E refer, respectively, to combined nonenols, suspensolide, bisabolene, anastrephin, and epianastrephin. Each bar represents the mean of two separate collections, made simultaneously from flies of the same cohort.

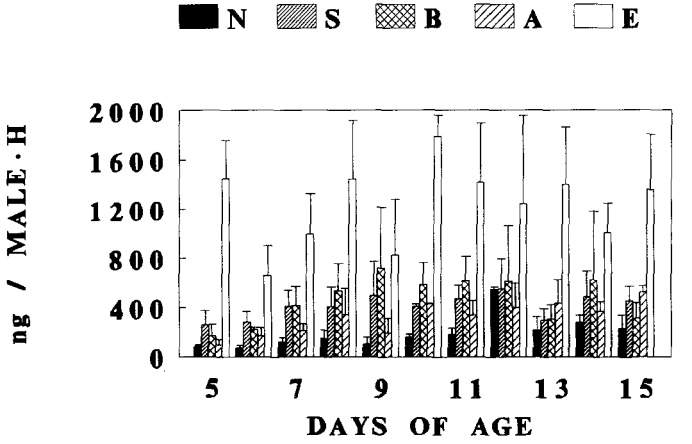

FIG. 4. Quantity (mean ± SD) and blend of volatiles released by a single male as a function of age. Males were taken at random from a large cage of males held in a 14 : 10 light–dark cycle. Collection was made during the 11th through the 13th hour of the photophase to include the hours of maximum release of volatiles. Values for most days represent six to eight collections from different males, with only a few representing as few as two collections. N, S, B, A, and E refer, respectively, to combined nonenols, suspensolide, bisabolene, anastrephin, and epianastrephin.

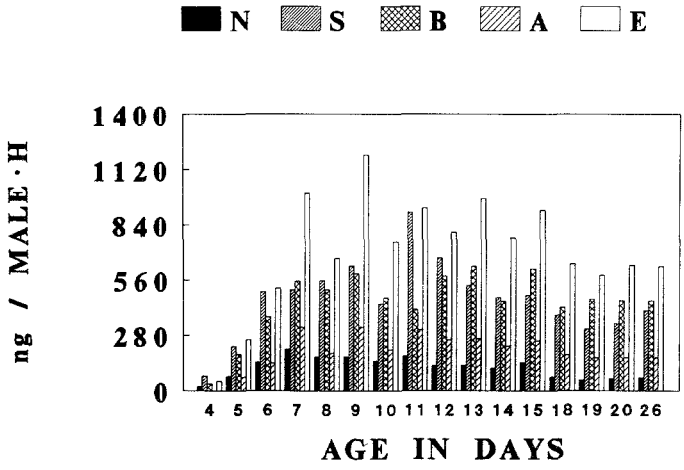

FIG. 5. Quantity (mean of two collections) and blend of volatiles released by a group of 25 males of *A. suspensa*. Males were kept on a 14:10 light-dark cycle. Volatiles were collected continuously for 21 hr at each age. N, S, B, A, and E refer, respectively, to combined nonenols, suspensolide, bisabolene, anastrephin, and epianastrephin.

prereproductive period as adults, started release of volatiles at about 5 days, but did not reach a maximum until they were 12–14 days old (Nation, 1989). Release of volatiles continued in old males; the oldest monitored were 35 days old, and they released (in nanograms per male per hour) nonenols 27, suspensolide 173, bisabolene 192, anastrephin 29, and epianastrephin 203.

Influence of Male Density on Quantity and Composition of Volatiles. The number of males in the group during collection of the volatiles influenced both the quantity and blend of the volatiles released. A single male released more total volatiles and more of each component than the per male average for a group of males (Figure 6, Tables 4 and 5). Differences in component ratios can also be seen in Figures 4 and 5, from single and multiple males, respectively. The reduction in anastrephin, epianastrephin, and nonenols by grouped males (32–37.5%, per male) is about twice as great as the reductions (14–16%) in suspensolide and bisabolene (Table 5). The minimum number needed to demonstrate the reduction in anastrephin or epianastrephin appeared to be about three individuals; a group of two males failed to show significant reduction, whereas three, five, and 10 males did show the reductions (Table 4).

Groups of males not only released less per male of components than a single male, but larger groups released less per male than smaller groups. Ten

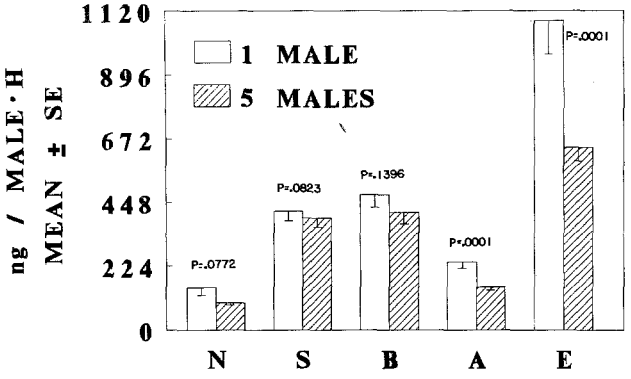

FIG. 6. Comparison of quantity of volatile components released by one male and the average for five males ($N = 25$). Male age varied from 6 to 21 days, but on any given day the single male and five males were the same age and were taken at random from a larger cage of males held on a 14:10 light-dark cycle. Probability values refer to the null hypothesis that the quantitative determination for a given component from the single male equals the value for the same component for the average of five males. N, S, B, A, and E refer, respectively, to combined nonenols, suspensolide, bisabolene, anastrephin, and epianastrephin.

or 15 males released less per male of anastrephin and epianastrephin than five males (Table 4). Apparently the difference between three and five males was not great enough, however, to result in detectable difference, or perhaps the flies did not detect a small difference in group size.

The possibility that a group effect might be demonstrated when females were present with a single male was tested. Collections ($N = 10$) from one male with four females did not result in reduction of any component in the volatiles, but the male was kept from mating by a separating screen because release of volatiles ceased during mating (Nation, 1989).

The relationship between epianastrephin and anastrephin collected from single males ($N = 116$ determinations) is shown in the linear regression data of Figure 7. Although the absolute quantities of anastrephin and epianastrephin released vary a great deal with group size, age, rhythm, cohort of flies, and individual tested, the ratio of epianastrephin to anastrephin was not influenced by any variables tested. In 52 determinations of volatiles collected from a single male the ratio was 4.7 ± 0.3 (mean $\pm$ SEM), while that for greater than one male collections (two, three, five or 10 males) was 4.5 ± 0.2 ($t = 0.54$, $P = 0.5905$).

TABLE 4. ANASTREPHIN OR EPIANASTREPHIN RELEASED BY SINGLE VS. SMALL GROUP OR SMALL GROUP VS. LARGER GROUP OF MALE *A. suspensa*

Individuals in test	*N*	Anastrephin (ng/male/hr)		*P*	Epianastrephin (ng/male/hr)		*P*
		Single male	Small group		Single Male	Small group	
1 vs. 2	12	192.5 ± 34.5	120.4 ± 15.3	0.0920	759.2 ± 129.9	515.9 ± 68.9	0.1297
1 vs. 3	10	247.6 ± 42.6	173.2 ± 20.1	0.1615	1240.9 ± 139.9	835.9 ± 71.0	0.0112
1 vs. 10	10	206.3 ± 21.7	135.6 ± 15.3	0.0189	870.6 ± 124.6	533.9 ± 64.2	0.0336
		Small group	Larger group		Small group	Larger group	
5 vs. 10	5	288.6 ± 34.2	200.2 ± 29.7	0.0777	1198.9 ± 103.6	862.5 ± 138.5	0.0496
5 vs. 15	7	219.8 ± 30.4	128.6 ± 7.3	0.0353	847.5 ± 83.5	559.7 ± 31.9	0.0273
3 vs. 5	5	223.6 ± 38.6	188.6 ± 14.1	0.3975	922.9 ± 36.8	853.1 ± 92.7	0.6914

[a] Pheromone was collected for 3 hr during the 11th through the 13th hr of photophase in a 14 : 10 light-dark cycle. Values are mean ± SEM.

TABLE 5. VOLATILES RELEASED BY SINGLE MALES OR GROUPED MALES COLLECTED DURING 11TH THROUGH 13TH HOUR OF PHOTOPHASE IN 14:10 LIGHT–DARK CYCLE[a]

Volatile component	Nanograms/male/hr		P	Group percent reduction
	Single males	Grouped males		
Nonenols	159.1 ± 14.9	108.0 ± 5.9	0.0008	32.1
Suspensolide	549.6 ± 28.8	457.3 ± 20.7	0.0032	16.8
Bisabolene	500.4 ± 33.7	430.5 ± 25.1	0.0452	14.0
Anastrephin	222.5 ± 15.3	143.3 ± 8.2	<0.0001	35.6
Epianastrephin	999.2 ± 70.7	624.5 ± 36.3	<0.0001	37.5

[a] Values are mean ± SEM; $N = 52$.

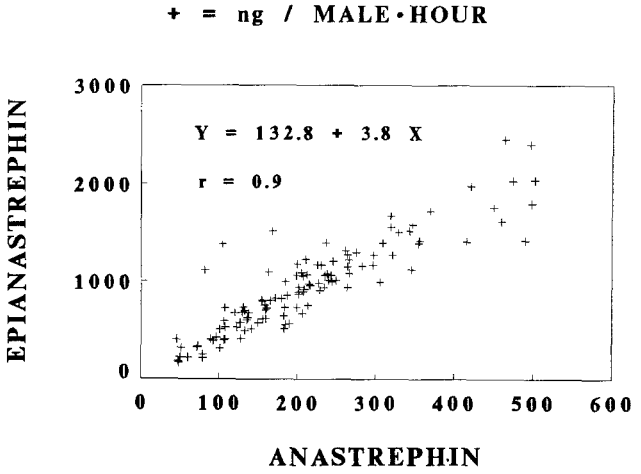

FIG. 7. The regression relationship for epianastrephin as a function of anastrephin in the pheromone blend released by single male flies ($N = 116$). Pheromone was collected during the period of maximum pheromone release (11–13 hr of the photophase) with flies on a 14:10 light–dark cycle. Flies varied in age from 6 to 21 days of age at collection.

DISCUSSION

Site of Release of Male Volatiles. The evidence presented supports the conclusion that volatile components are released from both mouth and anus of the male flies but does not entirely rule out the possibility that some volatiles are released directly through the cuticle. There are two male-specific glandular

tissues in male flies (Nation, 1974, 1981). These glands are the enlarged and highly modified salivary glands and large epidermal cells below the lateral abdominal pouches. Both glands show developmental changes correlated with sexual maturity in males (Nation, 1974). Gas chromatographic analyses of these and other tissues dissected from male flies showed that anastrephin, epianastrephin, and the nonenols were concentrated mainly in the rectal region of the hindgut, while suspensolide and bisabolene were absent or present only in very small quantities (Nation, 1989). Suspensolide, bisabolene, and small quantities of the nonenols and gamma lactones were present in the enlarged salivary glands of males. Suspensolide and bisabolene, but not nonenols and the gamma lactones, were present in pleural glands (enlarged epidermal cells) in males (Nation, 1989). Thus the distribution of pheromonal components in the rectum and in the salivary glands supports a release mechanism through the mouth and from the anus and/or anal pouch. Presumably, pleural gland cells could secrete components into and through the cuticle that lies immediately over the cells on the ventropleural abdomen.

Quantity and Blend Ratio in Volatiles. The total quantity of volatiles released by a single fly in an hour is very remarkable. Inspection of Table 5 shows that more than 2 μg/hr of total volatiles can be released. Only slightly more than 1 μg total was present in whole body extracts (Nation, 1989), so the males must synthesize the components rapidly as needed. This high level of synthetic activity can continue for a number of hours during an afternoon and, together with the physical display of puffing pouches and wing fanning, must be energetically very costly. Perhaps it should not be so surprising that males may, at times, adopt an alternative to the lek strategy (Burk, 1983), or that in groups they should find ways to reduce the quantity of pheromone released.

One constant feature of the collected volatiles was the ratio of epianastrephin to anastrephin (E/A). Anastrephin and epianastrephin are diasteriomers of each other (Battiste et al., 1983), and Chuman et al. (1988) suggested that they were derived from suspensolide or that they and suspensolide were derived from a common precursor. There is no obvious relationship indicated by the data of this report that allows one to choose between these possibilities. Another pattern that emerged was that the quantity of suspensolide and bisabolene in the volatiles was close to 1 : 1, with suspensolide coming out slightly greater than bisabolene in the data of Table 5. In collections on a given day, either might be the greater of the two, but they were always close. Preliminary analyses indicate that about 60% of the measured nonenols was (*Z*,*Z*)-3,6-nonadienol, and 40% was (*Z*)-3-nonenol.

Possible Role of Volatiles in Behavioral Ecology of Flies. Although Burk (1983) found that feral males under natural conditions in Homestead, Florida, actively courted females on fruit during the morning hours, he observed no matings during the morning. Late in the morning and during midday, both males

and females tended to rest underneath the leaves of (mostly) host trees. Male display behavior and territorial defense began to increase at about 1500 hr and peaked at 1700 hr, as did mating activity. The increase in display and mating activity in Burk's study in the field from 1500 to 1700 hr corresponds closely with a rhythm that is 10–12 hr into the photophase in the summer in Homestead and agrees with laboratory data from the present work on time of maximum pheromone release. Burk (1983) proposed, on the basis of his field observations, that males adopted, during the morning hours, an alternative to the lek strategy in which they attempted forced matings with females that were on the fruit and engaged in test borings or ovipositing. Data from this report show that males are releasing very low levels of pheromone during the morning hours and that the pheromone blend is very different early in the day compared to midafternoon and later in the day when most sexual activity and mating take place. Perhaps their courtship displays and the small amount of pheromone released, as well as the different blend, have important implications in the context of forced mating on the fruit during the morning, while the lek strategy requires its own set of parameters with respect to blend and quantity of pheromone released.

Selection of a male from a lek by females is expected to lead to selection for increasingly complex and elaborate courtship and/or display behaviors on the part of highly competitive males and even to establishing a rank order of males within a lek (Alexander, 1975; Emlen and Oring, 1977; Thornhill, 1980; Bradbury, 1981; Thornhill and Alcock, 1983; Burk, 1984). Thus males might be expected to try to outdo each other, and it might be expected that the mean per male pheromone output from a group of males would exceed the output from a single male. An unexpected result from this study was the discovery that groups of males reduce, on a per male basis, the quantity of all pheromonal components released in comparison to those released by single males. Reduction rather than stimulation of pheromone release seems inconsistent with the male competition theory. Perhaps a situation in which only some males might cease or greatly reduce volatiles production while letting others in the lek advertise and attract females to the vicinity is operating (Hendrichs, 1986). A selective advantage might accrue for males that ceased or greatly reduced pheromone production. The reduction in synthesis and release of pheromonal molecules may enable males to save energy, a savings that might enable a male to survive longer, release pheromone longer, release the most attractive blend longer, display longer, or broadcast a song (Webb et al., 1976, 1983, 1984) longer or louder, all of which may lead to increased chances of mating. Each male may be seeking to maximize chances for successfully mating and for survival. Unfortunately, no mechanism has been devised yet to collect volatiles separately from each member in a group in order to determine if only some individuals are reducing output.

It remains to be determined if observation of other sexual display characteristics, such as puffing of the body pouches or wing fanning, correlates closely enough with the actual quantity or blend of volatiles released to be of use in providing insight into pheromone output. McDonald (1987) has stated that there is intragroup stimulation of pheromone release, stimulation by sight of a mirror reflection, stimulation by sight of other males, and lack of stimulation by wingless flies of *Ceratitis capitata*, but in that work pheromone release was not measured chemically. Apparently the assumption was made that the puffing and other display characteristics can be used as indicators of pheromone release. I agree that displaying flies are releasing pheromone, but display behavior may not be indicative of the quantity being released. The display characteristics may serve functions other than, and in addition to, pheromone release, such as the establishment of male dominance within a lek.

It has been theorized that choosy females will benefit by selection of the male best fit to sire her offspring from among an assemblage of males in a lek (Bradbury, 1981, and references therein). The exact mechanism for female choice in Caribbean fruit flies is not known, although several parameters correlated with successful males are large size, display behavior, and sound production (Burk and Webb, 1983; Sivinski, 1984; Hendrichs, 1986). Because the pheromone blend released varies continuously with age and time of day, it seems unlikely that females choose a male based upon either blend or quantity of pheromone released. The role of the pheromone may be to attract females to the lek site, whereupon other close-range mechanisms, possibly involving pheromone as well as sound (Webb et al., 1976, 1984) and vision, play roles. Recent field trapping of flies with sticky traps baited with the total volatiles mixture collected as in this report indicated that both laboratory-reared and released, and feral Caribbean fruit flies were attracted to the volatiles in a trap (data to be published elsewhere). Tests in the field with individual components or combinations have not been conducted.

All data reported in this paper have come from observations on laboratory-reared flies. While it has been shown that wild flies do release the same components and in similar proportions (Nation, 1989), it is now well known that long-established laboratory cultures of insects usually show differences from wild populations. It is important to determine the responses of wild flies, particularly in density-dependent situations that may more nearly mimic a true lek situation than the present glass cylinders do. Males of several tephritid fruit flies release pheromonal volatiles, some with characteristic body and behavioral displays similar to those reported for Caribbean fruit fly males (Feron, 1959; Lhoste and Roche, 1960; Pritchard, 1967; Malavasi et al., 1983; Morgante et al., 1983; Robacker and Hart, 1985a,b), but detailed collections and analyses of the volatiles, or of factors influencing pheromone release, have not been reported.

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EFFECTS OF PREDATOR FECAL ODORS ON FEED SELECTION BY SHEEP AND CATTLE[1]

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Abstract—The effectiveness of predator fecal odors in modifying feeding selection by sheep and cattle was investigated in two trials. In trial 1, animals could select from feed bins contaminated with coyote, fox, cougar, or bear fecal odor, and oil of wintergreen, or select the control feed. All odors were rejected ($P < 0.01$) by sheep and cattle, except bear odors by sheep. In trial 2, animals could select feed during 10-min periods in an open 11-m × 16-m arena. Fecal odor did not influence approaches to feed bins, or head entries into bins. Only coyote fecal odor reduced ($P < 0.05$) the time spent feeding in the contaminated bin, and increased ($P < 0.05$) consumption from the control bin by both cattle and sheep. Some animals on some test days refused to feed from either feed bin, although cattle and sheep closely inspected bins. Results suggest that fecal odors may not prevent livestock from entering a treated area but may reduce the time spent grazing in such an area.

Key Words—Feeding behavior, predator odors, feces, cattle, sheep.

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INTRODUCTION

Chemical signals have been used to modify herbivore feeding behavior, usually to protect crops or tree plantations (Müller-Schwarze, 1983; Melchiors and Leslie, 1984; Campbell and Bullard, 1972; Conover, 1987). Predator odors have modified feeding by black-tailed deer (*Odocoileus hemionus columbianus*) (Müller-Schwarze 1972, 1983; Melchiors and Leslie, 1984; Sullivan et al., 1985a), snowshoe hares (*Lepus americanus*) (Sullivan et al., 1985b), voles (*Microtus* spp.) (Sullivan et al., 1988a), and pocket gophers (*Thomomys talpoides*) (Sullivan et al., 1988b). Müller-Schwarze (1972) suggested that black-tailed deer and possibly other ungulates possess innate negative responses to predator odors.

Olfaction by domestic livestock such as sheep (*Ovis aries*) or cattle (*Bos taurus*) is important in feeding (Arnold, 1966; Arnold and Hill, 1972; Arnold et al., 1980). Livestock may also react negatively to odors (Arnold and Hill, 1972). Engle and Schimmel (1984) noted that steers were marginally displaced from a preferred grazing site because of peripheral application of a deer repellent. Poindron (1974) reported that dog (*Canis familiaris*) feces repelled feeding sheep. Although predator fecal odors have repelled grazing ungulates such as deer, it is uncertain whether livestock would also be repelled either by smell (Arnold et al., 1980) or novelty (Chapple and Lynch, 1986) rather than fear of a potential predator (Sullivan et al., 1985a). Gregarious livestock species probably depend more upon visual and auditory acuity or vigilance (Ydenberg and Dill 1986) than upon olfactory cues for predator defense. For example, Hutson (1982) reported that flight distance in sheep decreased as group size increased.

Our research was designed to determine if food selection by sheep and cattle was affected by predator fecal odors. We are interested in the potential use of repellents for application to large patches of toxic larkspur (*Delphinium barbeyi*) plants (Ralphs et al., 1988). Grazing cattle ingest large amounts of tall larkspur when foraging in larkspur-dominated vegetation areas (Pfister et al., 1988a,b). We compared the responses of cattle and sheep to assess species differences.

METHODS AND MATERIALS

Trial 1. Fecal material was collected from bear (*Ursus americanus*), cougar (*Felis concolor*), fox (*Vulpes vulpes*), and coyote (*Canis latrans*) housed in zoos. Animal diets were a variable mixture of red meat, commercial feeds, and vegetables. Fecal material was collected fresh each morning, and frozen immediately at −20°C. Trials involved five Holstein steers each weighing 255 kg and five Targhee sheep each weighing 60 kg. The steers had no previous expe-

rience with predators, and the sheep may have had prior contact with coyote scat, but probably had no prior contact with the excreta of other predators.

Steers and sheep were individually housed under shelter in 3 × 4-m and 1.5 × 3-m pens, respectively. All animals were fed an ad libitum diet of alfalfa hay pellets in their pens for 14 days before trials began. Two feed bins were attached to the pen wall 3 m and 2 m apart for cattle and sheep, respectively. A preliminary 10-day trial determined whether there was any position bias in feed selection (Elliott and Loudon, 1987).

Feed bins were contaminated with fecal odor by placing fecal material in 20 × 6 × 6-cm aluminum pans fitted with wire-mesh lids, and pans were wired into position 30 cm above the bottom of the feed bin. This did not alter the animals' ability to feed from the bin, but ensured that fecal odors were in close proximity to feed. Fecal material was thawed at room temperature beginning 12 hr before use and mixed with water (4 : 1) in a Waring blender. Approximately 75 ml of the resultant slurry was added to the pans. During odor trials of 72-hr each, feed intake from each bin was monitored at 1, 6, 24, 48, and 72 hr (Arnold et al., 1980), except cattle intake was not monitored at 1 hr. The containers holding the fecal material were recharged and bin position changed at 0, 12, 24, 36, 48, and 60 hr. Control bins were treated similarly but no fecal material was added. Consecutive 72-hr trials were conducted using the fecal material; an additional trial using cotton wool saturated with oil of wintergreen was added as a novel control odor (Arnold et al., 1980). Feed consumption from contaminated feed bins was expressed as a percentage of total feed eaten (contaminated + control bins), following the procedures of Elliott and Loudon (1987).

Trial 2. Trial 2 determined whether cattle and sheep responded to odors when feeding in a open arena. The 16-m × 11-m outdoor arena had a dirt floor, and sides of metal panels covered with heavy canvas. There was a feed bin in each of two corners, 14 m apart. Animals were habituated to the arena by feeding alfalfa pellets from a large feeder for three days. Animals were fed at 2% of body weight (maintenance ration) and could consume their daily ration in about 1 hr. Before their daily feeding for the next eight days, animals were individually fed a limited amount of pellets for 10-min periods each morning from the experimental feeders. The animals learned to quickly move from one feeder to another to maximize pellet consumption.

Fecal material was treated as described above when placed in the pan in the treated bin; an empty pan was wired in the control feeder. Other pans with and without fecal material were placed 1 m apart at ground level in an L-shape around the treated and control bins, respectively, so animals had to pass a pan in order to approach the bin. To avoid a position bias, the fecal and control treatments were alternated daily. After an overnight fast, animals were tested each morning for two consecutive days. The sheep or cattle were individually

monitored for 10-min periods in the area. Measured variables were the number of approaches to the control or treated bin, the number of distinct head entries into the bins, the time spent feeding in each bin, and pellet consumption from each bin.

Statistical Analysis. For trial 1, confidence intervals (99%) were calculated based on consumption during the preliminary trial to correct for position bias. This confidence interval is feed consumed from the contaminated bin expressed as a percentage of total feed eaten from both the control and contaminated bin, and was calculated from results of the 10-day preliminary trial. Consumption of pellets during the trials at levels below the 99% confidence limit indicate rejection due to odor treatment (Elliott and Loudon, 1987). For trial 2, feed consumption from treated and control bins was analyzed by ANOVA techniques, using animals as blocks ($N = 5$) with repeated measurements over days ($N = 6$) (Gill, 1978). Confidence intervals (95%) were used to test differences in approaches, entries, and time spent feeding at each bin.

RESULTS

Trial 1. Cattle and sheep exhibited no significant position bias (Table 1). Both cattle and sheep responded ($P < 0.01$) to fecal odors and to oil of wintergreen (Table 2) by decreasing feed consumption from the treated bin. However, fecal odors were ~4–20× more effective than oil of wintergreen in reducing consumption. Bear feces was not effective with sheep ($P > 0.01$), but was effective with cattle ($P < 0.01$). There was little evidence of adaptation to coyote, cougar, or fox odors over the 72-hr periods (Figure 1). However, both cattle and sheep clearly adapted to bear feces and oil of wintergreen (Figure 1).

Trial 2. Animals did not alter their approaches to the bins because of treatment ($P > 0.05$), nor did the fecal odors affect the number of head entries into the bins ($P > 0.05$) (Table 3). Coyote fecal odor influenced ($P < 0.05$) the

TABLE 1. MEANS AND CONFIDENCE LIMITS FOR PERCENTAGE OF TOTAL FEED CONSUMPTION FROM FEEDERS DURING ADAPTATION PERIOD

	Feed consumption (%)			
	Position			
	1	2	SE	99% CI
Sheep	44	56	2.12	50 ± 7.1
Cattle	52	48	1.04	50 ± 5.6

TABLE 2. EFFECTS OF FECAL ODORS ON FEED CONSUMPTION BY 5 SHEEP AND 5 CATTLE OVER A 72-hr PERIOD

	Consumption (kg)			
	Sheep		Cattle	
Treatment	Total	Treated bin (%)	Total	Treated bin (%)
Coyote	44.12	2.24 (5.1)	142.44	3.14 (2.2)
Bear	46.46	20.18 (43.4)	114.28	26.06 (22.8)
Fox	45.32	1.87 (4.1)	117.99	9.19 (7.8)
Cougar	48.97	2.36 (4.8)	107.76	11.65 (10.8)
Oil of wintergreen	46.46	19.07 (41.0)	113.89	48.85 (42.9)
99% confidence interval (% of feed consumed)		(42.9–57.1)		(44.4–55.6)

time sheep and cattle ate from the bins (Table 3). Although trends were similar for all fecal odors, there were no other significant differences ($P > 0.05$) because of the high variability.

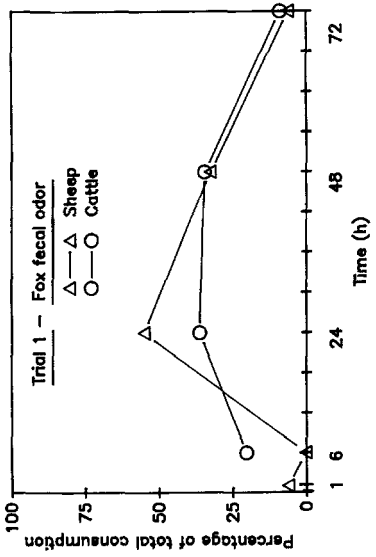
Consumption was affected by day ($P < 0.05$) for cattle, but not for sheep ($P = 0.15$). There were day × treatment interactions ($P < 0.05$) for both cattle and sheep responses. Only coyote fecal odor decreased ($P < 0.05$) pellet consumption (Table 3, Figure 2); cattle and sheep responses to fox and cougar fecal odors were not consistent over days.

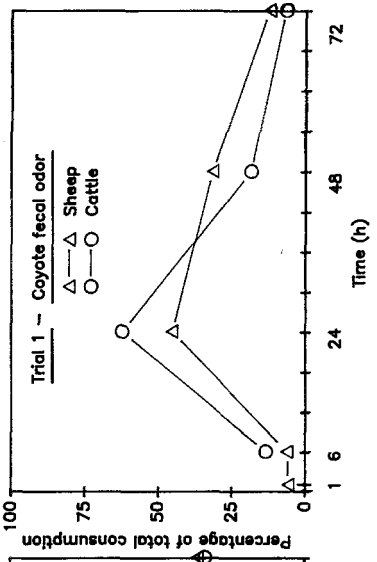
DISCUSSION

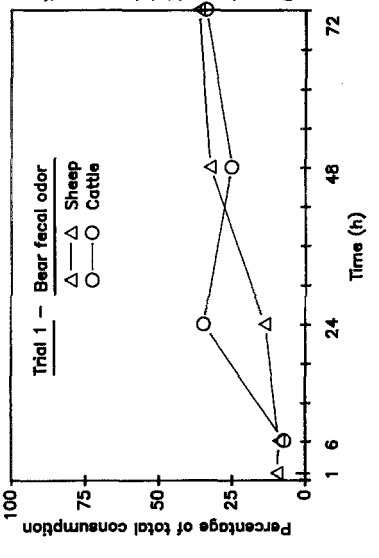
Cattle and sheep responded negatively to fecal odors during trial 1. Livestock disliked feeding in close proximity to coyote, fox, and cougar feces. There was less response to bear fecal odors, and sheep and cattle differed in their responses. Sullivan et al. (1985a) reported that coyote, fox and cougar feces suppressed deer feeding, while Melchiors and Leslie (1984) found that cougar but not coyote feces inhibited deer feeding.

Oil of wintergreen was much less effective in deterring feeding than most types of fecal material, which indicates that animal response was not due simply to novel odors. Both oil of wintergreen and fecal odors were novel, but most fecal odors caused greater avoidance. Arnold et al. (1980) found that oil of wintergreen reduced feed consumption by sheep. Unlike the methods of Arnold et al. (1980), oil of wintergreen did not directly contact the feed in our study.

There was no evidence of habituation to those fecal odors that deterred feeding. In contrast, Arnold et al. (1980) found that sheep habituated rapidly


Trial 1 – Fox fecal odor
Sheep
Cattle
Percentage of total consumption
100
75
50
25
0
1 6 24 48 72
Time (h)


Trial 1 – Coyote fecal odor
Sheep
Cattle
Percentage of total consumption
100
75
50
25
0
1 6 24 48 72
Time (h)


Trial 1 – Bear fecal odor
Sheep
Cattle
Percentage of total consumption
100
75
50
25
0
1 6 24 48 72
Time (h)

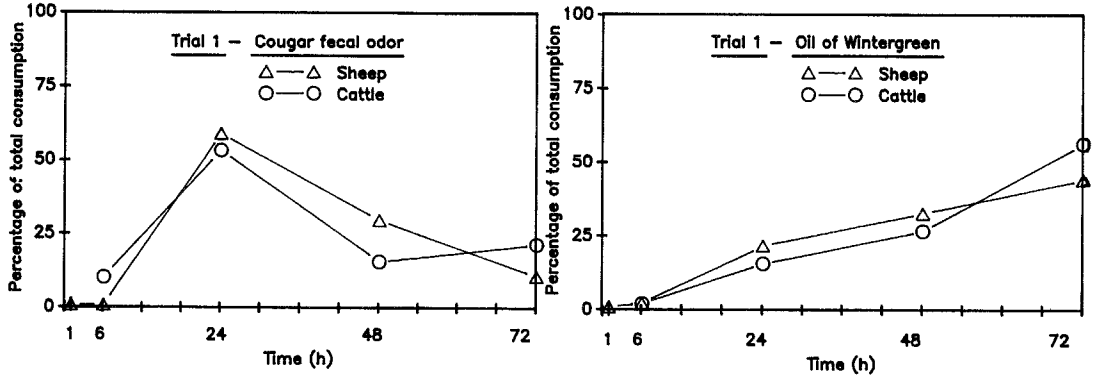


FIG. 1. Consumption of feed during 72-hr test periods by sheep and cattle from feed bins treated with predator fecal material. Feed consumption from the treated bin is expressed as a percentage of total feed consumed from the treated and control bins at each point in time.

TABLE 3. MEAN FREQUENCY OF ANIMAL MOVEMENT DURING 10-min PERIODS IN RELATION TO FEED BINS, AND TIME SPENT EATING IN FEED BINS

	Cattle			Sheep		
	Fecal material					
	Coyote	Fox	Cougar	Coyote	Fox	Cougar
Approaches to bin[a]						
Control	1.9a[b]	2.3a	2.1a	3.0a	1.3a	1.7a
Fecal	1.3a	1.4a	1.4a	1.8a	1.7a	1.5a
Head entries into bin						
Control	6.9a	5.1a	4.2a	4.8a	1.9a	4.1a
Fecal	2.2a	1.8a	1.2a	2.2a	2.0a	2.0a
Time spent feeding (sec)						
Control	273.6a	155.3a	201.9a	235.6a	102.8a	221.5a
Fecal	15.1b	45.8a	40.7a	11.8b	83.7a	109.8a
Intake per feeding period (g)						
Control	372.9a	165.6a	180.3a	270.9a	100.4a	159.6a
Fecal	19.3b	76.8a	62.4a	21.1b	96.8a	119.2a

[a] Approaches to bin indicates animals moved through a line of pans set at ground level containing fecal material.

[b] Means in the same column for the same variable and type of fecal material with the same letter are not different ($P > 0.05$).

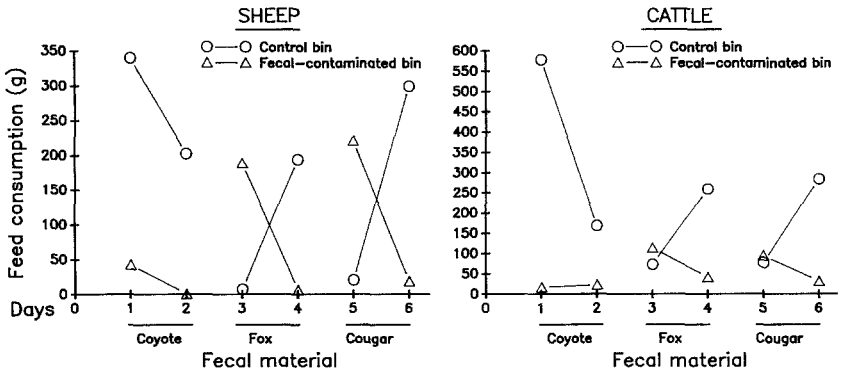


FIG. 2. Mean consumption (g) of alfalfa pellets during 10-min testing periods by cattle and sheep from control and treated feed bins in a large arena.

to chemical odors. In the mornings, most of the sheep bedded down on the opposite side of the pen from the bin contaminated with coyote, cougar, or fox feces, an indication of a severe aversion to the odors.

Sheep and cattle tended to respond similarly over time to coyote, cougar, and fox feces (Figure 1). Peak consumption was reached at 24 hr, with consumption declining over the rest of the period. We are unable to explain this trend.

In both trials, livestock investigated the odors in the bins. During trial 2, animals quickly entered the arena and would stop suddenly and sniff carefully when they approached the pans containing the feces. The animals would then usually approach the feed bin. As Sullivan et al. (1985a) and Müller-Schwarze (1972) noted, deer closely examined predator feces. This behavior may be characteristic of other ungulates.

Coyote feces deterred feeding by both cattle and sheep in the large arena trial. This material was also highly deterrent to both species in the small pen trial. The active compound(s) that elicited this response is unknown. These trials also gave no indication whether the response is innate or if the predator odors are deterrent independent of selective pressures. Coyote predation in sheep flocks in the western United States has a serious detrimental impact on sheep, but predation in cattle herds is probably small (Taylor et al., 1979).

Results of our trials suggest that feces may not deter livestock from entering an area contaminated with fecal material. However, the odor may cause grazing livestock to reduce the amount of feeding time spent in an area because of noxious olfactory stimuli. Engle and Schimmel (1984) reported that cattle use of preferred grazing areas was reduced by application of a commercial deer repellent around the periphery of the areas. Müller-Schwarze (1983) also noted that fecal odors reduced the amount of time that deer foraged in gardens. During trial 2, one sheep and one steer refused to eat from either bin during the last four test days. Even though they were very hungry, they simply wandered around the arena. Two other sheep and steers also exhibited this avoidance behavior for at least two days of the trial. When livestock did eat during trial days 3–6, they often appeared to be confused when they entered the arena and eventually went to a favored position, regardless of treatment. This position bias, absent during the coyote fecal trial (day 1 and 2), surfaced during the last four trial days with cougar and fox feces, producing the trends found in Figure 2.

Research trials in which feces or other repellents are applied under grazing conditions seem warranted. These studies should examine the efficacy of semiochemicals for modulating livestock feeding on toxic, but otherwise palatable plants, and how responses are influenced by environmental variables and time. Research should also determine the efficacy of deterrents in reducing livestock browsing damage in agroforestry situations (Knowles and Tahan, 1979). The

use of synthetic compounds derived from predator feces would greatly enhance research efforts and practical application. Abbott et al. (1989) have developed a synthetic repellent from active compounds in African lion (*Panthero leo*) feces that has been effective in preliminary trials.

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REPELLENCY OF VOLATILE FATTY ACIDS PRESENT IN FRASS OF LARVAL YELLOW MEALWORMS, *Tenebrio molitor* L. (COLEOPTERA: TENEBRIONIDAE), TO LARVAL CONSPECIFICS

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Abstract—Frass of late-instar larvae of *Tenebrio molitor* L. contained 0.0889 g of butyric acid, 0.0279 g of propionic acid, and 0.0175 g of valeric acid per 100 g. Grouped larvae were strongly repelled by butyric acid at the 10^{-1} M concentration. Lower concentrations of butyric acid were less repellent. Valeric acid was repellent at 10^{-1} M to 10^{-3} M concentrations, below which no tested concentration, including one identical to that occurring in prepared solutions of frass found to be attractive, displayed any effect. Propionic acid was repellent at the concentration in prepared solutions of frass, which were strongly attractive. Concentrations of 10^{-1} M, 10^{-3} M, and 10^{-4} M propionic acid were also found to be repellent. The implications of the repellency of these compounds to groups of mealworm larvae are discussed, with particular reference to the interaction between these frass components and others that have already been studied.

Key Words—Repellency, volatile fatty acids, propionic acid, butyric acid, valeric acid, yellow mealworm, *Tenebrio molitor*, Coleoptera, Tenebrionidae.

INTRODUCTION

Infestations of the yellow mealworm, *Tenebrio molitor* L., are generally found in damp, dark places where cereals may be decaying (Cotton, 1963). These localized regions may contain large amounts of frass from several generations

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of *T. molitor*. Experiments have been conducted testing mealworm frass, and lactic and acetic acids, which are frass components, for behavioral effects (Weaver et al., 1989; Weaver and McFarlane, 1989). The frass and lactic acid acted as aggregants, and acetic acid had no behavioral influence (Weaver et al., 1989). However, the chemical analysis of *Tenebrio* frass indicated the presence of volatile fatty acids in significant amounts, as were found in the frass of other omnivorous insects (McFarlane and Alli, 1985). McFarlane (1984) found that frass volatile fatty acids were repellent to *Blattella germanica*. Lactic acid in the frass of *Blattella* was found to be aggregative (McFarlane and Alli, 1986). These results indicated a variety of responses were possible with these chemical stimuli in the frass, with one component being dominant. In addition, propionic acid has been found to aggregate *Acheta domesticus* (McFarlane et al., 1983) and butyric acid has been identified as an aggregant for *Periplaneta americana* (McFarlane and Alli, 1987). The present article deals with the behavioral responses caused by volatile fatty acids in the frass of larval *Tenebrio molitor*. In particular, experiments were conducted using serial dilutions of volatile fatty acids to determine activity at various concentrations. Experiments were also conducted using concentrations of acids identical to those occurring in behaviorally active frass samples. These frass samples, and lactic acid occurring in them, were found to be attractive (Weaver et al., 1989). Bioassay indicated the isolated volatile fatty acids were repellent. A mixture of volatile fatty acids and lactic acid was prepared to determine how the activity of these isolated components of frass was manifested in the natural environment, where they occur concurrently. Volatile fatty acids 10^{-1} M were also evaluated for olfactory responses in an active airflow olfactometer.

METHODS AND MATERIALS

Yellow mealworms, *Tenebrio molitor* L., were raised on a diet of wheat bran, whole wheat four, and brewer's yeast (50:45:5 w/w) at 25 ± 1°C and 55 ± 5% relative humidity and a photoperiodic regime of 14:10 light–dark. The density of all stages per filled 4.55-liter glass culture jar was not allowed to exceed 250 individuals. Collection of frass and diet, determination of volatile fatty acids, and the bioassay arena in both were identical to those used in Weaver et al. (1989).

The test protocol involved a choice test using Whatman No. 1 filter papers treated with 0.68 ml of test chemical in aqueous solution at the appropriate concentration versus a paper treated with 0.68 ml of water (distilled, deionized in both cases). The papers were allowed to dry 1.5 hr prior to testing. Dried papers were fixed in the arena opposing each other, and 20 yellow mealworms (140–160 mg) were introduced simultaneously. The papers were examined at

0.5-hr intervals after the initiation of the trial, and larvae on each paper were counted, if they were actually on the paper or were touching it with their antennae, mouthparts, or legs. Trials were terminated after 5 hr. Reagent grade acids (Anachemia Limited, Montreal, Canada) were used in preparation of all test solutions.

An active airflow olfactometer (as in Weaver and McFarlane, 1989) was used to evaluate the mode of chemoreception for these repellent chemicals. The test stimuli were derived from two shredded filter paper strips treated with 0.68 ml of a 10^{-1} M aqueous volatile fatty acid solution and evaporated 0.5 hr prior to bioassay. Control stimuli were similarly prepared using filter paper strips treated with 0.68 ml distilled water.

Statistical comparisons between the various test stimuli were based on analysis of variance using Tukey's HSD test on mean aggregation indices (AI) calculated by subtracting the number of larvae on the control paper from that on the treated paper and dividing by the total number on the two papers. Index values may range from -1 (complete repellancy) to $+1$ (all responding individuals on the treatment paper). A value of -0.2 corresponds to 50% more insects on the control paper and -0.33 corresponds to twice as many insects on the control as were on the treatment paper (Roth and Cohen, 1973). Indices were based on ten observations per replicate, with 12–20 replicates being conducted per test chemical. Indices were analyzed by *t* test for significant differences from a value of zero (SAS Institute, 1982), which would represent identical numbers on treatment and control papers. Analysis of the responses to the grouped olfactometer bioassay were based on a one-tailed test using Wilcoxon's signed-ranks test for paired observations to determine if the number of insects responding to the chemical stimulus was significantly less than the number responding to the control.

RESULTS

Frass of late instar larvae of *Tenebrio molitor* L. contained 0.0889 g of butyric acid, 0.0279 g of propionic acid, and 0.0175 g of valeric acid per 100 g. These acids were not present in the diet.

The responses of late-instar *T. molitor* to serial dilutions of those acids present in the frass and one not occurring in the frass but chemically similar (isobutyric acid) are shown in Table 1. Valeric acid at 10^{-2} M and 10^{-3} M was most strongly repellent. Two unusual effects were evident as well. The values of 10^{-2} M propionic acid and 10^{-2} M isobutyric acid were both distinct from those for the concentrations surrounding them. Propionic acid at 10^{-2} M had a much higher (though statistically similar) AI value and showed no significant repellency at all. Both 10^{-3} M and 10^{-1} M propionic acid are significantly

TABLE 1. RESULTS OF TRIALS USING SERIAL DILUTIONS OF FRASS ACIDS AND ISOBUTYRIC ACID

Acid	Concentration	Mean AI (±SE)[a]	Significance[b]
Butyric	10^{-1} M	$-0.34 \pm .03^{efg}$	0.001
Butyric	10^{-2} M	$-0.22 \pm .03^{de}$	0.001
Butyric	10^{-3} M	$-0.04 \pm .03^{abcd}$	NS
Butyric	10^{-4} M	$-0.11 \pm .04^{abcd}$	0.01
Butyric	10^{-5} M	$-0.12 \pm .05^{abcd}$	0.05
Propionic	10^{-1} M	$-0.17 \pm .05^{bcde}$	0.001
Propionic	10^{-2} M	$-0.03 \pm .05^{abcd}$	NS
Propionic	10^{-3} M	$-0.23 \pm .03^{def}$	0.001
Propionic	10^{-4} M	$-0.23 \pm .04^{def}$	0.001
Valeric	10^{-1} M	$-0.17 \pm .04^{cde}$	0.005
Valeric	10^{-2} M	$-0.49 \pm .03^{g}$	0.001
Valeric	10^{-3} M	$-0.43 \pm .03^{fg}$	0.001
Valeric	10^{-4} M	$0.03 \pm .04^{a}$	NS
Isobutyric	10^{-1} M	$0.04 \pm .03^{a}$	NS
Isobutyric	10^{-2} M	$-0.17 \pm .04^{bcde}$	0.001
Isobutyric	10^{-3} M	$0.06 \pm .04^{a}$	NS

[a] Means followed by the same letter are not statistically different at $P \leq 0.05$.
[b] Results of t test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P \leq$ the value listed. NS indicates responses to treatment and control papers are not statistically different.

repellent. Isobutyric acid was significantly repellent at 10^{-2} M and caused no effect at either 10^{-1} M or 10^{-3} M.

The responses of grouped yellow mealworms to the amounts of isolated frass acids found in 20 mg frass/ml H_2O (13.6 mg frass/paper), as tested in Weaver et al. (1989), are shown in Table 2. Propionic acid was the only repellent chemical (when tested in isolation) found in this solution of frass, which had been found to exhibit overall attractancy (Weaver et al., 1989).

The olfactometer bioassay of 10^{-1} M concentrations of the volatile fatty acids indicated they were significantly repellent to groups of large *Tenebrio* larvae. These results are shown in Table 3.

A mimic solution composed of all acids (volatile fatty acids + lactic and acetic acids) isolated from the frass at the concentration in which they occurred in attractive 20 mg frass/ml H_2O gave an AI value of 0.35 ± 0.04. This showed significant attraction at the $P \leq 0.001$ level (based on 12 replicates).

Figure 1 shows the response of yellow mealworms to repellent 10^{-3} M propionic acid throughout the bioassay. Trial stabilization occurred rapidly and a large decrease in mealworms on the treatment and control papers occurred at 4.5 hr (consistent through 12 replicates).

Figure 2 shows the response of yellow mealworms to 10^{-3} M butyric acid,

TABLE 2. RESULTS OF TRIALS USING DILUTIONS OF FRASS ACIDS EQUIVALENT TO THOSE IN ATTRACTIVE CONCENTRATION OF WHOLE FRASS EXTRACT (20 mg Frass/ml H_2O)

Acid	Amount (g)[a]	Mean AI (±SE)[b]	Significance[c]
Butyric	1.2×10^{-5}	-0.05 ± 0.04^a	NS
Propionic	3.8×10^{-6}	-0.22 ± 0.04^b	0.001
Valeric	2.5×10^{-6}	0.04 ± 0.03^a	NS

[a] Total amount of acid applied to filter paper in 0.68 ml of solution. Concentrations of solutions used were: butyric acid, 2.0×10^{-4} M; propionic acid, 7.5×10^{-5} M; valeric acid, 3.6×10^{-5} M.

[b] Means followed by the same letter are not statistically different at $P \leq 0.05$.

[c] Results of t test for significant response to treated paper, i.e., a significant difference from zero (equal numbers on treatment and control). All indices are significant at $P \leq$ the value listed. NS indicates responses to treatment and control papers are not statistically different.

TABLE 3. RESULTS OF OLFACTOMETER TRIALS USING VOLATILE FATTY ACIDS

Acid (10^{-1} M)	Treatment	Control	T_s value[a]	Significance[b]
Butyric	11	41	0	0.025
Valeric	7	49	0	0.025
Propionic	4	50	0	0.025

[a] Wilcoxon's signed-ranks test for paired observations, all differences of like sign (the lowest value of $D = 0$).

[b] Treatment significantly less than control at $P \leq$ the given value. Calculations based on responding individuals for six replicates of 10 larvae each.

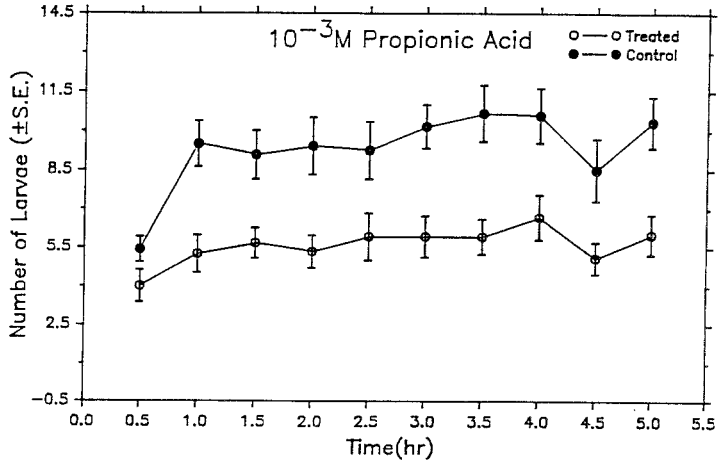

FIG. 1. Mean number of mealworm larvae (±SE) responding to papers treated with 0.68 ml of 10^{-3} M propionic acid and dried 1.5 hr. Based on 12 replicates of 20 larvae each.

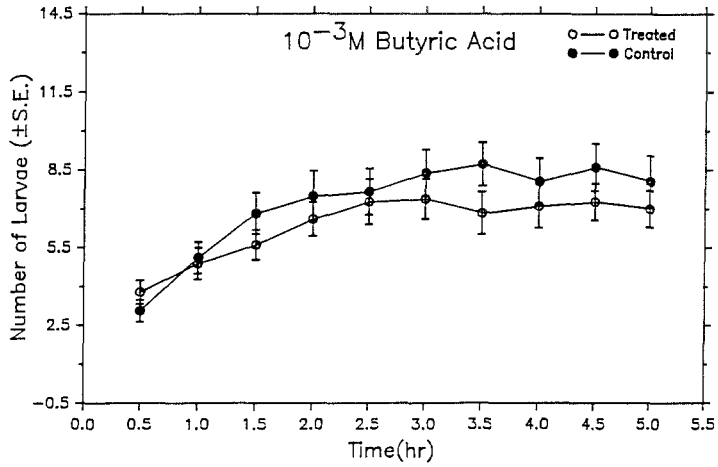

FIG. 2. Mean number of mealworm larvae (±SE) responding to papers treated with 0.68 ml of 10^{-3} M butyric acid and dried 1.5 hr. Based on 18 replicates of 20 larvae each.

a noneffective concentration, throughout the duration of the bioassay. The response occurred much more slowly in this case than it did for a repellent chemical (approximately 2.5 hr to have 75% of the insects responding versus 1.0 hr in Figure 1).

DISCUSSION

The analyses of frass and diet samples indicate that these volatile fatty acids are produced in the larval mealworms, since they are not found in the diet but are present in the frass. These chemicals may therefore be utilized as discriminants of areas that were previously inhabitated by conspecific larvae.

The bioassays conducted suggest that relatively high concentrations of these volatile acids are required to significantly repel yellow mealworms. In fact, only propionic acid is active at the same concentration as in biologically active frass solutions. The effect, is in apparent contradiction with that induced by aqueous extracts of frass containing the same amount of isolated acid, since frass extracts and their lactic acid component have been previously demonstrated to be attractive/arrestant (Weaver et al., 1989). Therefore, a bioassay was conducted using the precise concentration of synthetic volatile fatty acids + lactic and acetic acids that occurred in active frass preparations, with the results indicating strong attraction to this mimic solution. These volatile fatty acids are present in frass samples in 1/3 to 1/10 of the amount (g) that the lactic and acetic acids were

observed in the frass (Weaver et al., 1989). Therefore, it is not surprising that the overall response to a solution of volatile fatty acids + lactic and acetic acids designed to mimic an attractive frass stimulus (equivalent to 20 mg frass/ml H_2O) was aggregative.

However, there are factors other than concentration involved. Earlier experiments have established that lactic acid in the frass of *Tenebrio* does not have an airborne influence on behavior, but is likely to be perceived through contact chemoreceptors (Weaver and McFarlane, 1989). This was probably because lactic acid has very low volatility and probably does not volatilize from dilute aqueous solutions or the surface of fecal pellets. These volatile fatty acids, however, did display repellent effects when tested on groups of larvae in an olfactometer at higher concentrations. It is evident that the larval mealworms must come into contact with the filter paper to aggregate in response to frass extracts or isolated lactic acid (Weaver and McFarlane, 1989), so it is likely that initial exploration is directed towards substrate evaluation, although this might be overcome by repellent airborne messages. It may also be likely that the concentration of airborne molecules from a treated filter paper or fecal pellet may not exceed the behavioral threshold until a larva is extremely close to it. The amount of volatile fatty acid on the paper (or arising from the fecal particles) and that at any given distance from source would decrease by volatilizing and subsequently dissipating with the passage of time. The attractive component is not subject to this reduction in quantity and can remain strongly attractive. Therefore, frass in the environment can exert a pronounced behavioral influence through contact, without the prolonged manifestation of the repellency of these low concentration volatile components. This was similar to the effect noted by McFarlane (1984) when isolated volatile fatty acids in concentrations occurring in the frass were found to be repellent to larvae of the German cockroach, but frass-conditioned papers and certain components of the frass were attractive. It is likely that the attractive chemical (lactic acid in both species) elicited a dominant behavioral response.

Therefore, the preference of the larvae for the control papers in these trials may involve an initial rejection of the substrate (through close range repellency) and then aggregation on the control paper, rather than only responding to a continuous repellent stimulus. Weaver and McFarlane (1989) have demonstrated an innate tendency for mealworms to aggregate on control papers in clusters of higher than expected densities, which means individuals may attract each other. Bioassays determining the orientation of animals that occur in groups naturally should be based on the responses of groups primarily and subsequently on the factors mediating this grouping. The existence of conspecifics in these bioassays in no way invalidates the significance of grouped larvae being repelled from a stimulus in these experiments.

Comparison of Figures 1 and 2 illustrates that trial stabilization required

more time in those cases where the insects showed no orientation to a particular chemical. This was also observed in our earlier experiments with certain non-attractive concentrations of lactic acid (Weaver et al., 1989). This may indicate that both repellent and attractive chemicals serve to orient *T. molitor* larvae more rapidly to suitable locations for cluster formation.

The preceding discussion centers on the nature of the interaction between the components in a given quantity of frass. We feel that this readily supports our hypothesis that frass acts as a chemical marker of safe refugia for these slow-developing insect larvae (Weaver et al., 1989). However, the rapid accumulation of fresh frass from a relatively high density of mature larvae may result in a considerable airborne concentration of volatile fatty acid. The resulting repellency could be of considerable ecological benefit, since large numbers of these relatively large slow-developing larvae might well have exhausted the available foodstuffs in that particular region. This would be the situation where larval dispersion might be the most beneficial. In addition, we have demonstrated that high concentrations of lactic acid are also repellent (Weaver et al., 1989), which might also occur under such conditions. Therefore, the repellent chemical message may be further enhanced by the probable epideictic (Prokopy, 1981) nature of lactic acid. Regardless of the relative role of the individual chemicals, very high concentrations of frass are likely to produce chemical messages that differ from those of more moderate amounts.

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SUPPRESSION OF OVIPOSITION IN *Oryzaephilus surinamensis* (L.) (COLEOPTERA: CUCUJIDAE) FOLLOWING PROLONGED RETENTION IN HIGH-DENSITY CULTURES OR SHORT-TERM EXPOSURE TO LARVAL VOLATILES[1]

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Abstract—After 20 days in a high-density culture containing many larvae, female sawtoothed grain beetles, *Oryzaephilus surinamensis* (L.), laid half as many eggs in a 24-hr oviposition bioassay as females held for six days in the same culture, or for six or 20 days in a low-density culture. Oviposition by females held for six days in a high-density culture was reduced to a similar extent when they were exposed in the oviposition bioassay to an oat flake treated with an extract of Porapak Q-captured larval volatiles (equivalent to 5000 larval hours). A retained suppression of oviposition rate after prolonged exposure to larvae or an induced reduction caused by short-term exposure to larval volatiles both could be of adaptive advantage in reducing the risk of oviposition in an already densely populated habitat.

Key Words—*Oryzaephilus surinamensis* (L.), Coleoptera, Cucujidae, population density, culture age, semiochemicals, oviposition, larval volatiles.

INTRODUCTION

Population density has many complex influences on insects that exploit exhaustible food resources. For example, some insects release epideictic (spacing) pheromones that mitigate against excessive population density by promoting

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intraspecific dispersion (Prokopy, 1981). In other species, fecundity may be reduced at high population densities (Peters and Barbosa, 1977).

The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.), is a cosmopolitan pest of stored products. Feeding male *O. surinamensis* produce macrolide aggregation pheromones (Pierce et al., 1985, 1987), a class of pheromones given the trivial name cucujolide because of its prevalence in the family Cucujidae (Oehlschlager et al., 1988). Pheromone production rates by young *O. surinamensis*, as well as olfactory responses to pheromones and food volatiles, are sensitive to the density of conspecifics (Pierce et al., 1983, 1989).

Observations of mature, dense laboratory cultures of our strain of *O. surinamensis* revealed the presence of very few larvae per female, suggesting that oviposition may be suppressed by exposure to high-density cultures. We tested this hypothesis by investigating the oviposition response by *O. surinamensis* in relation to culture age, adult density, and the presence of larval volatiles.

METHODS AND MATERIALS

Maintenance and Handling of Experimental Insects. O. surinamensis were maintained on large-flake, rolled oats and brewer's yeast (95/5, w/w) in 3.8-liter glass jars at 28–30°C and 40–60% relative humidity in darkness (Pierce et al., 1981). Adults and larvae were separated from oat flakes with a sieve and collected by aspiration.

Collection of Larval Volatiles. Larval volatiles were obtained by aerating larvae (ca. 10,000) without food in a 2-liter Erlenmeyer flask at 24°C in darkness (Pierce et al., 1981, 1985). Charcoal-filtered, humidified air was drawn by aspiration (1.65 liters/min) over the larvae and then through a glass trap (150 × 14 mm OD) filled with Porapak Q (50–80 mesh, Applied Sciences Division, Milton Roy Laboratory Group, State College, Pennsylvania). An aeration was continued with the same Porapak Q trap for 480 hr, using fresh larvae and a clean chamber for each 24-hr period. Mixed-sex larvae (predominantly last instar) for aerations were harvested 20 days after inoculation of fresh medium with 24,000 adults (mixed age and sex) per kilogram of medium. Volatiles were recovered by extraction of the Porapak Q with purified pentane in a Soxhlet extractor for 24 hr and concentrated by distilling off the pentane through a Dufton column. The amount of volatiles collected was expressed as larval hours (lh) where 1 lh = the volatiles from one larvae in 1 hr.

Oviposition Bioassay. Mixed-sex cultures were set up with 800 or 24,000 adults/kg of medium. To obtain females for bioassay from cultures without larvae and pupae, beetles were removed six days after culturing, just before the first eggs were ready to hatch. For females from cultures with larvae and pupae,

beetles were removed 20 days after culturing, just before the first adults of the next generation were about to emerge.

For each test, beetles (23–40 days posteclosion) were removed from a culture, immediately sexed (Halstead, 1963), and 20 females per treatment (i.e., 20 replicates) were put individually into the bottoms of 20, 1-dram glass vials, each containing one oat flake. Polyethylene vial stoppers (with five small holes punctured for ventilation) were put on, and the vials were placed in darkness at 30°C and 65–75% relative humidity for 24 hr. Fresh oat flakes had been conditioned at 30°C and 65–75% relative humidity for 24 hr prior to bioassay. In tests where the oat flakes were treated, a 10-μl aliquot of either larval volatiles in purified pentane or purified pentane as a control was applied to an oat flake, the solvent allowed to evaporate, and the oat flake and a female immediately placed in the vial as above. After 24 hr the females were removed from the vials, and all eggs were counted under a dissecting microscope.

Experiments. In the first of two experiments, females that had spent six or 20 days in either low-density cultures (800 adults/kg) or high-density cultures (24,000 adults/kg) were tested in the 24-hr, oat-flake, oviposition bioassay. In the second experiment, females were removed from high-density cultures after six or 20 days and tested in the oviposition bioassay for response to one of three treatments: an untreated control oat flake, a pentane-treated (solvent control) oat flake, and an oat flake treated with a pentane extract of 5000 lh of Porapak Q-captured larval volatiles. The data were analyzed by ANOVA followed by the Newman-Keuls test.

RESULTS

In all cases, no larvae or pupae were present in 6-day-old cultures. Conversely, larvae and pupae were always present in 20-day-old cultures (Tables 1 and 2).

After 20 days in a high-density culture, female *O. surinamensis* laid significantly fewer eggs than females removed from the same culture after six days or removed from low-density cultures after six or 20 days (Table 1).

In the second experiment, *O. surinamensis* females from a 6-day-old, high-density culture laid significantly fewer eggs in the presence of an oat flake treated with a pentane extract of Porapak Q-captured larval volatiles than did females exposed to pentane-treated and untreated control oat flakes (Table 2). Females removed from the 20-day-old culture with larvae and pupae laid significantly fewer eggs on untreated or solvent control oat flakes than females removed from the same culture after six days (Table 2), confirming the results of the first experiment. Exposure of these females to an oat flake treated with larval vola-

TABLE 1. OVIPOSITION RESPONSE IN 24-HR BIOASSAY BY *O. surinamensis* REMOVED FROM LOW- AND HIGH-DENSITY CULTURES BEFORE AND AFTER LARVAE AND PUPAE WERE PRESENT (N = 20 REPLICATES)

Culture density (adults/kg of medium)	Days in culture medium	Presence of larvae and pupae in culture	Eggs/female ($\bar{X} \pm$ SE)[a]
800	6	−	3.85 ± 0.49a
800	20	+	3.35 ± 0.41a
24,000	6	−	3.70 ± 0.31a
24,000	20	+	1.65 ± 0.29b

[a]Means followed by same letter are not significantly different, Newman-Keuls test, $P < 0.05$.

TABLE 2. EFFECT OF PORAPAK Q-CAPTURED LARVAL VOLATILES ON OVIPOSITION RESPONSE BY *O. surinamensis* REMOVED FROM HIGH-DENSITY CULTURES (24,000 ADULTS/KG OF MEDIUM) BEFORE AND AFTER LARVAE AND PUPAE WERE PRESENT (N = 20 REPLICATES)

Days in culture medium	Presence of larvae and pupae in culture	Treatment of oat flake	Eggs/female ($\bar{X} \pm$ SE)[a]
6	−	none	3.30 ± 0.24a
6	−	pentane	3.20 ± 0.21a
6	−	5000 lh	1.80 ± 0.24b
20	+	none	1.50 ± 0.24b
20	+	pentane	1.40 ± 0.28b
20	+	5000 lh	1.30 ± 0.22b

[a]Bioassays for 6- and 20-day females conducted in separate 24-hr sessions. Means followed by same letter are not significantly different, Newman-Keuls test, $P < 0.05$.

tiles, however, did not cause a statistically significant decrease in their oviposition rate (Table 2).

DISCUSSION

Different mechanisms may be involved in suppression of oviposition in females removed after 20 days from high-density cultures (Tables 1 and 2) and those removed after six days and exposed to larval volatiles (Table 2).

The induced suppression of oviposition in the latter females by the odor of

larvae is apparently a response to a pheromonal signal indicating a well-populated habitat. Another example of a larval egg-laying deterrent occurs in *Spodoptera littoralis* (Boisduval), for which an aqueous extract of larval feces suppressed oviposition to about the same degree as in our experiments (Hilker, 1985). There are various examples of epideictic (marker) pheromones (Prokopy, 1981) emitted by eggs, larvae, pupae, and adults of other insect species (Prokopy et al., 1984). These pheromones apparently serve an immediate, density-regulating function in habitats (unlike stored grain) that cannot easily support large larval populations. Similarly, the antiaggregation pheromones produced by bark beetles indicate a saturated habitat (Borden, 1985).

Additionally, we have provided evidence indicating retained suppression of oviposition as a consequence of prior residence in a crowded population. In the females exposed to high-density cultures for 20 days, suppression of oviposition was retained for at least the 24-hr period of the bioassay. This suppression might be the result of a physiological feedback mechanism that inhibits vitellogenesis in response to the odor of immatures or some other component of a high-density culture. (Alternatively, it might be a learned habituation caused by prolonged exposure to larval odor.) It could not occur as a result of physiological fatigue following oviposition, because females removed from low-density cultures after 20 days retained the capability for maximal oviposition (Table 1). Like the pheromonal suppression of oviposition, the retained suppression following long-term exposure to immatures could be of adaptive advantage in deterring oviposition in a potentially befouled habitat.

We have been unable to duplicate the results in Table 1 with two strains of the merchant grain beetle, *O. mercator* (Fauvel), although there was an apparent weak, but insignificant, suppression of oviposition by females in one strain held for 26 days in a high-density culture. Thus, our results with *Oryzaephilus* spp. parallel those with *Tribolium* spp. in which net fecundity was only slightly reduced in the confused flour beetle, *T. confusum* du Val, maintained in flour previously occupied by adults, whereas it was reduced to a greater extent in the red flour beetle, *T. castaneum* (Herbst), in previously occupied medium (Prus, 1961). In populations of *T. castaneum*, however, there exists genetic variability for the responsiveness of egg production to the quality of the environment (Lavie et al., 1978), and this genetic character is maintained in natural populations by a balance between the selective advantage of high responders in optimal environments and their selective disadvantage in suboptimal environments (Lavie et al., 1981).

O. surinamensis and *O. mercator* have a taxonomically close relationship (Slow, 1958). Differential habits of resource utilization may contribute to species specificity. Based on a model proposed for *Tribolium* spp. (Ghent, 1963; Ziegler, 1976), we previously have suggested that *O. surinamensis* and *O. mercator* might perform different ecological roles as colonists of temporary habitats

(Pierce et al., 1983, 1987). The more dispersive *O. surinamensis* may be a primary colonist suited to exploit widely distributed, sparsely populated, and very fresh habitats, whereas *O. mercator* may be a secondary colonist better able to invade and persist in partially depleted habitats already occupied by populations of the same or different species.(Pierce et al., 1983). Sensitivity of aggregation pheromone production (Pierce et al., 1989) and perception (Pierce et al., 1983) to overcrowding by conspecifics in *O. surinamensis* supports our model, as does the strong cross-attractiveness of an *O. surinamensis*-produced cucujolide to *O. mercator* (Pierce et al., 1987). The oviposition response demonstrated by *Oryzaephilus* spp. is consistent with this model. Female *O. surinamensis* might restrict the number of eggs laid in depleted habitats already heavily occupied with larvae and adults by either a long-term, retained suppression of oviposition, or through the detection of an epideictic pheromone. Alternatively, our present results and past studies (Pierce et al., 1983, 1987, 1989) suggest there are little or no means by which *O. mercator* can detect and avoid a partially depleted habitat. Since *Oryzaephilus* spp. lay a predetermined number of eggs in a lifetime (Howe, 1956), it could be of definite adaptive benefit for females of a pioneering, colonizing species such as *O. surinamensis* to delay oviposition until a fresh or sparsely inhabited resource is located.

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FIELD TEST OF THE PHEROMONE HYPOTHESIS FOR HOMING BY PACIFIC SALMON

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Abstract—Experimental groups of juvenile coho salmon (*Oncorhynchus kisutch*) were released to elucidate the relative importance of site-specific ("imprinted") odors and intraspecific odors ("pheromones") in homing. Adult salmon returned to their release site rather than a hatchery containing both adult and juvenile salmon of their population. Furthermore, salmon sharing the same parents returned to different sites, suggesting that local movements are not strongly influenced by siblings or other conspecifics.

Key Words—Coho salmon, *Oncorhynchus kisutch*, homing, imprinting, olfaction.

INTRODUCTION

It is generally accepted that salmonid fishes use olfaction to locate their natal river in the final stages of homing (Wisby and Hasler, 1954). However, it is not clear which odors convey the identity of the river. It has been hypothesized that the fish are guided by chemicals from riverine features such as rocks, soil, and plants, which create a unique combination of odors (Hasler and Scholz, 1983). On the other hand, Nordeng (1971, 1977) propounded the hypothesis that population-specific odors ("pheromones") emanating from both juvenile conspecifics residing in freshwater and those migrating to sea (smolts) guide the homing adults. Studies with artificial odorants (e.g., Scholz et al., 1976) indicated that pheromones were not necessary for homing. However, recent laboratory studies have indicated that salmonids can discriminate between populations based on chemical cues (Groot et al., 1986; Olsen, 1986; Quinn and Tolson, 1986; Stabell, 1987). Moreover, coho salmon (*Oncorhynchus kisutch*)

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can also distinguish siblings from nonsiblings (Quinn and Busack, 1985; Quinn and Hara, 1986). There is currently no evidence that discrimination of kin- or population-specific odors is involved in homing.

Larkin (1975) expanded upon Nordeng's (1971) hypothesis that odors from juveniles guide returning adults and hypothesized that salmon from a given population might associate with each other while at sea on their homeward migration. Larkin's hypothesis stemmed from the mathematical demonstration that errors in group orientation decrease as school size increases, if the individuals jointly orient to a mean direction (Larkin and Walton, 1969). Thus if salmon schooled with members of their population, migration might be more efficient.

To investigate the relative importance of site-specific odors and odors emanating from conspecifics in homing, coho salmon were reared under experimental conditions, marked, and released. The subsequent patterns of homing by adult salmon from the experimental groups were monitored. The study posed two specific questions: (1) Will adult coho salmon pass a site containing both adults of their population and juveniles directly related to them (full siblings) to reach an upriver site where they had been released? (2) Will the homing behavior of one group of salmon influence that of a genetically related group returning at the same time to a different site within a river system?

METHODS AND MATERIALS

Incubation and Treatment of Juveniles. Between November 18 and 25, 1985, adult coho salmon from the University of Washington population were spawned and the fertilized eggs divided into two groups. No parents were common to both groups or to any other juvenile coho produced that season. All embryos were incubated at the University of Washington (UW) hatchery using dechlorinated Seattle city water, a source not generally used in the hatchery. Previous experiments (Whitman et al., 1982; Quinn et al., 1983) demonstrated that adult salmon can distinguish between city water and hatchery water. Water temperatures averaged 7–9°C and the eggs hatched in early January 1986. Development of hatchlings ("alevins") continued until yolk sac absorption was complete in March.

When the fry were free-swimming, they were moved to the Seward Park hatchery on Lake Washington (Figure 1) (March 8 for group 1 and March 21 for group 2). Both groups remained at this site in circular ponds, rearing in water pumped from the lake directly into the hatchery. Small individuals were culled from group 1, and 9489 fish were marked by excision of the adipose fin and internal coded wire tags on June 16. Group 2 was culled and divided into two subgroups (2A: $N = 10{,}020$ and 2B: $N = 10{,}148$) for fin-clipping and tagging on June 13–14. The salmon began the process of transformation from

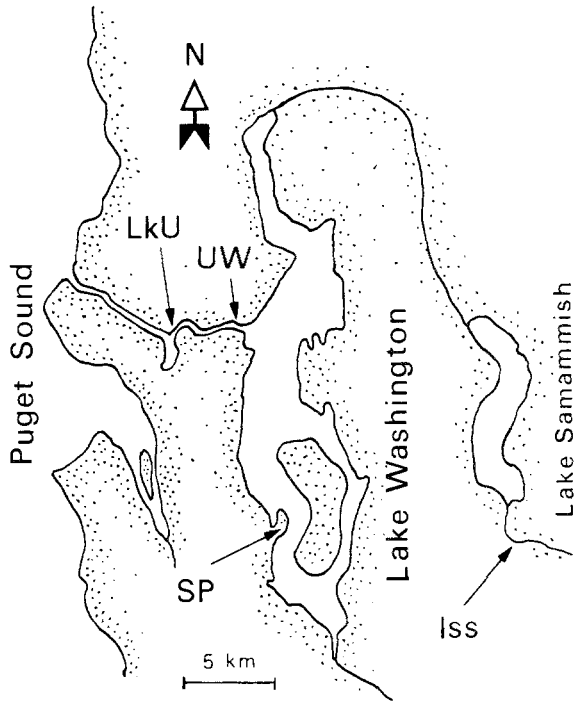

FIG 1. Map of the Lake Washington watershed showing the locations of the release site on Lake Union (LkU) and the hatcheries at the University of Washington (UW), Seward Park (SP), and Issaquah Creek (Iss).

freshwater "parr" to marine-adapted "smolts" in late June, judged by silvery appearance and downstream swimming behavior. On July 8, 1986, the tagged members of group 1 were released from the Seward Park hatchery into Lake Washington. Approximately 4000 untagged coho from group 1 were retained at the hatchery. On June 27, the tagged fish from group 2B were released from Seward Park hatchery. On July 1, group 2A fish were trucked from Seward Park to a site on Lake Union about 2.2 km below the UW hatchery (Figure 1) and released. The release site was a boat launching ramp with no suitable spawning area in the vicinity. Table 1 summarizes the treatments which the three groups received.

Collection of Returning Adults. Salmon released as smolts in 1986 were expected to return as adults primarily in fall 1987. This population has relatively few precociously maturing males, known as jacks (Brannon et al., 1982). Jacks could have entered the UW hatchery return pond in fall 1986 and would have been detected in the regular surveys taken three times each week. The trap at

TABLE 1. COHO SALMON EXPERIMENTAL TREATMENTS, INDICATING WHEN (IN 1986) FISH WERE EXPOSED TO DIFFERENT WATER SOURCES OR MOVED AND SITE TO WHICH THEY RETURNED[a]

Developmental stage or operation	Experimental group		
	Group 1	Group 2A	Group 2B
Eggs, alevins, fry			
Site	CW	CW	CW
Parr			
Site	SP	SP	SP
Date	3/8	3/21	3/21
Tagging (smolts)			
Site	SP	SP	SP
Date	6/16	6/13	6/14
Release			
Site	SP	LkU	SP
Date	7/8	7/1	6/27
Size	13.9 g	12.6 g	11.3 g
Returns			
Site	SP/UW	SP/UW	SP/UW
Nos.	39/0	6/32	43/0

[a] CW refers to dechlorinated city water at the University of Washington hatchery, SP refers to the Seward Park hatchery, LkU refers to the Lake Union release site and UW refers to the University of Washington return pond.

the return facility at Seward Park was not constructed until late summer 1987, however, and returning jacks would not have been collected there in 1986.

Dechlorinated city water, used to incubate the eggs and embryos, was not released from the UW hatchery at any time during the spawning season. The adult coho (and chinook, *O. tshawytscha*) salmon holding in the UW hatchery's pond in the fall presumably produce a high concentration of salmon odors in the effluent. These odors were supplemented by the 4000 immature siblings from group 1, which were transferred from Seward Park to the UW hatchery prior to adult returns.

All salmon returning to the UW and Seward Park hatcheries were inspected for marks. Additionally, three other opportunities for recovering tagged fish were pursued. The U.S. National Marine Fisheries Service (NMFS) has a small hatchery across the ship canal from the UW hatchery. While no NMFS fish were expected to return in 1987, the hatchery's trap was checked for stray experimental salmon. The Washington Department of Fisheries operates a large

hatchery for chinook and coho salmon on Issaquah Creek, a tributary to Lake Samammish above Lake Washington (Figure 1). Coho salmon returning to this facility were checked for coded wire tags. Additionally, a gillnet fishery in the north end of Lake Washington and in Lake Samammish targets Issaquah Creek hatchery salmon. Subsamples from this fishery were taken by the Washington Department of Fisheries and checked for tags.

RESULTS

Thirty-nine salmon from group 1, incubated in dechlorinated city water and reared in Seward Park water, bypassed the UW hatchery containing full-sib relatives and other adults of their population and returned to Seward Park. No members of this group entered the UW hatchery. Similar behavior was shown by group 2B coho with a similar rearing history: all 43 recoveries were at the Seward Park hatchery (Table 1). However, 32 salmon (including one jack in 1986) from group 2A, reared at Seward Park but trucked to Lake Union for release (Figure 1) returned to the UW hatchery and only six adults returned to Seward Park.

Fifteen coho salmon entered NMFS trap but none were from any of the experimental groups. No coho salmon from any of the three groups were recovered from the fishery in Lake Washington or at the Issaquah hatchery.

DISCUSSION

Salmon returning to Seward Park had to swim within about 100 m of the UW hatchery. The UW hatchery contained full-sibling relatives of group 1 as well as 1708 adult coho (and 506 chinook) salmon over the course of the spawning season. In spite of these odors in the hatchery's effluent, salmon from groups 1 and 2B swam past the UW hatchery and approximately 16 km in Lake Washington to Seward Park. The tendency of salmon to return to a site experienced as juveniles rather than to one containing conspecifics' odors is consistent with the attraction of adult coho salmon to hatchery water without coho odors over city water containing juvenile coho in a two-choice apparatus (Brannon et al., 1984). Our result is also consistent with the demonstration by Black and Dempson (1986) that Arctic char were not decoyed into a nonnatal river by the presence of adults.

Laboratory experiments have indicated that coho salmon are capable of population-specific and family-specific chemosensory discrimination (Quinn and Busack, 1985; Quinn and Hara, 1986; Quinn and Tolson, 1986; Courtenay, 1989). Intraspecific discrimination may be related to juvenile social behavior

or mate selection by adults, but it apparently is not crucial to homing as all adults swam past the UW hatchery to Seward Park.

The return of all members of group 2B to Seward Park corroborated the results with group 1: pheromones or other population-specific odors in the UW hatchery did not attract them. Moreover, the fish in group 2B were the full siblings of those in 2A. The return of 2A to the UW hatchery (32 of 38 adults) indicated that homing salmon were not influenced by the behavior of siblings. This particular experiment was motivated by Larkin's (1975) hypothesis that homing salmon might school preferentially with kin at sea and by the idea that the local movements of adult salmon in freshwater are influenced by density of conspecifics (Quinn and Fresh, 1984). While our results provided no evidence for kin-based schooling, the tendency of group 2A to enter the UW hatchery was noteworthy. These fish were never reared in UW hatchery water and were moved to Seward Park prior to the presumptive time of olfactory imprinting (Hasler and Scholz, 1983) on the same schedule as groups 1 and 2B.

The return migration of group 2A might be explained by the following scenario which assumes that smolts imprint on a sequence of odors during emigration (Quinn et al., 1989, in press). The smolts learned the characteristics of Seward Park water and the odors of the release site but were denied the opportunity to experience the waters in between. Upon return to the watershed, they located the release site in Lake Union. However, from that point they could not detect Seward Park effluent odor, and they lacked experience of the homeward path. In this situation the fish were decoyed to the UW hatchery, the site that either most resembled the odors of home or contained familiar salmon odors (Brannon and Quinn, 1989). Some of the group 2A salmon (16%) were apparently more persistent in their search and returned to Seward Park. Thus this study indicates that salmon odors were not used to guide salmon home, although in the absence of primary cues they may facilitate the selection of spawning sites by straying salmon.

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RESPONSES TO INTER- AND INTRASPECIFIC SCENT MARKS IN PINE MARTENS (*Martes martes*)

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Abstract—In order to analyze intra and interspecific olfactory discrimination, behavioral responses (sniffing and marking) towards various odors were observed in pine martens (*Martes martes*). Two adult males and two adult females were tested for intraspecific discrimination of abdominal gland odors and urine. Both sexes sniffed and marked objects carrying their own scent less than unscented objects. There were no differences in sniffing or marking objects impregnated with odors from known and unknown conspecifics of the opposite sex or objects carrying the odor of an unknown male or female. A second experiment with two adult females gave no evidence for interspecific discrimination: no differences emerged when comparing reactions towards marks of pine martens, stone martens (a closely related species), or genets. The most obvious result of this study is the reduced response of pine martens towards their own mark. It is suggested that scent marking in martens may reflect "autocommunication," the primary effect being to familiarize an animal with its environment.

Key Words—Carnivores, pine martens, scent marking, odor discrimination, *Martes martes*.

INTRODUCTION

Studies on olfactory communication in carnivores (Rasa, 1973; Gorman, 1976; Roeder, 1980; Macdonald, 1985) have shown that scent marking has diverse social functions: scent marks are indicators of individual identity and carry information about sexual and social status as well as physiological state. Pine martens (*Martes martes*) show two types of marking: secretions from the

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abdominal gland and urine. Based on field observations, Pulliainen (1981a) has stated that "pine martens recognize the smells of other martens individually" and has suggested that "the pine marten also has its distinctive smell, which serves for interspecific recognition" (pp. 1085–1086).

The aim of the present study was to observe martens' reactions towards various odors in order to clarify the possibility of intra- and interspecific scent discrimination and thus analyze the social significance of scent marking in this species, which has been studied only rarely (e.g., Pulliainen 1981a,b; 1982). The first experiment investigates the reactions of martens towards various conspecific scent marks, while the second analyzes responses towards other species' scent marks.

METHODS AND MATERIALS

Experiment 1: Intraspecific Discrimination. In this experiment, the ability of martens to discriminate between their own scent mark and those of known and unknown males and females was tested. The study was carried out on two adult females and two adult males kept separately in outdoor cages (3 × 2.50 × 2.70 m), each containing shelters, tree trunks, and a water bowl. Every day the animals were fed dead chickens, fruits, and cake. Fresh water was available ad libitum.

The reaction of each individual to various conspecific scent marks was observed. The different scent marks were collected on plastic boxes (12 × 10 × 10 cm) that were introduced into the donor's cage the night preceding the test. Concurrent observations (mean of 3 hr/day/animal) on social interactions allowed us to verify that the test boxes were marked by the donors. Each animal was tested in the presence of (1) control boxes (unscented), (2) its own scent marks, (3) an unknown male's marks, (4) an unknown female's marks, (5) a known male's marks for females and a known female's marks for males. A known conspecific is defined as an individual living in visual, auditory, and probably olfactory contact with the subject (i.e., separated by a double wire mesh).

Each test consisted of placing the object in the animal's enclosure and recording the total duration of sniffing the object, the number of times the object was marked and the number of marking acts elsewhere in the cage. This last measure was taken to test whether general scent marking activity was modified by introducing the boxes. Each test lasted 10 min. (Previous observations of 30 min showed that 5 min after the introduction of the box, the animals no longer showed interest in the object.) A series of tests consisted of presenting the five stimuli to one individual in random order. Nine series of tests were completed

for each male, and nine and five, respectively, for each female, the discrepancy being due to different activity levels in the two females. The durations of sniffing and marking frequencies in the different odor conditions were compared with those recorded during control tests using Mann-Whitney U tests.

Experiment 2. Interspecific Discrimination. In this experiment, the ability of two female martens to discriminate between scent marks of their own and other carnivore species was investigated. This study was conducted on two adult females that had been maintained together for three years but separated four weeks prior to the study. They lived in indoor cages (3.20 × 2.50 × 1.90 m). The floor was covered with sawdust. The light cycle of 12 hr light (0700–1900 hr) and 12 hr dark was inverted. The martens were fed mainly on mice but were also given supplements of fruits and eggs.

The two females' responses to the following objects were observed: (1) unscented, (2) animal's own mark, (3) known female conspecific's mark, (4) stone marten's (*Martes foina*) mark, (5) genet's (*Genetta genetta*) mark. Stimuli 4 and 5 were used to compare reactions towards marks of a closely related species (stone marten) and a distantly related species (genet). The different scent marks were collected on bricks (15 × 6 × 3 cm) previously left in the donor's cage for several days. The procedure was the same as in experiment 1, the only difference being that each test lasted 5 min. Ten series of tests were completed for each subject. Statistical analyses were done as in experiment 1.

RESULTS

Experiment 1. The results are shown in Table 1. The typical behavior of martens towards novel objects consisted of sniffing the object and then scent marking on it. The most frequent type observed was abdominal marking; urination accounted for only 14% of all marking acts in females and 22% in males. The most obvious result for all four animals was that the control box was sniffed significantly longer and marked more frequently than the object carrying the test animal's own scent ($P < 0.05$). No statistical differences were observed between control objects and any other condition (known and unfamiliar, male or female), except for males reacting to the scent of known females: the duration of sniffing was longer with the known female's odor ($P < 0.05$), but the rate of marking did not differ.

There were no significant differences in responses to marks of known and unknown conspecifics of the opposite sex, either in males or in females. In females there were no significant differences in responses to the scent marks of an unknown male and an unknown female. Similarly, males did not behavior-

TABLE 1. MEAN DURATION OF SNIFFING (TOTAL/NUMBER OF 10-MIN PERIODS) AND MEAN MARKING FREQUENCIES (TOTAL NUMBER/NUMBER OF 10-MIN PERIODS) IN FEMALES AND MALES ON OBJECTS CARRYING VARIOUS ODORS AND IN SUBJECT'S CAGE

	Females			Males		
Conditions	Mean duration of sniffing (sec)	Mean frequency of marking on the objects	Mean frequency of marking in the cage	Mean duration of sniffing (sec)	Mean frequency of marking the objects	Mean frequency of marking in the cage
Unscented	32.7 ± 27.7 [a]	2.78 ± 1.20 [a]	2.35 ± 3.17	[a] 37.5 ± 18.7 [a]	2.61 ± 1.23 [a]	7.27 ± 3.55
Animal's own mark	11.2 ± 4.5	0.92 ± 1.09	1.28 ± 2.05	14.8 ± 7.4	1.16 ± 1.70	6.83 ± 4.12
Known female's mark				57.5 ± 25.7	2.83 ± 3.05	8.61 ± 4.04
Unknown female's mark	26.9 ± 16.0	2.42 ± 1.44	3.28 ± 4.60	46.1 ± 23.6	2.16 ± 2.81	7.66 ± 4.77
Known male's mark	21.2 ± 11.9	2.35 ± 2.37	2.42 ± 2.89			
Unknown male's mark	27.3 ± 17.6	1.85 ± 1.99	2.28 ± 2.88	46.1 ± 20.9	2.22 ± 1.90	7.88 ± 4.39

[a] $P < 0.05$.

ally differentiate between the scent marks of an unknown female and those of a strange male.

Comparing the various test conditions, no significant change in marking activity elsewhere in the enclosure was noted. Therefore, the reactions observed towards the various scent marks appear to be specific to objects carrying the different scents.

Experiment 2. As in experiment 1, the object carrying the animal's own mark was sniffed and marked significantly less than the unmarked object (Table 2, $P < 0.05$). Female A never marked the object carrying her own scent. No statistical differences appeared either when comparing control objects with other odors or when comparing reactions (sniffing duration and marking activity) towards marks of pine martens, stone martens, or genets.

DISCUSSION

The most clear-cut result of this study concerns reactions of pine martens towards their own marks. In all the tested animals, the scent-marked object was always sniffed and marked less than unscented objects, and there were no differences in reactions to unscented objects and objects carrying scent marks from a conspecific or from other species, except in males. Pulliainen (1982) argued that in pine martens scent marking has a "self-centered primary function" by playing a role in orientation and familiarity with a locality. Both experiments 1 and 2 suggest that pine martens recognize their scent marks as being their own, since the animals respond little to the presence of their own mark (this being particularly significant considering the marking rate of the animals). Another explanation would be that the animals were "blind" to marked objects and simply mark every object that contrasts with the general olfactory background. However, this explanation appears untenable since differences were found when males were confronted with known females' scent marks. Nevertheless, these two interpretations would lead to the same conclusion, namely, that scent marking could play a role in the maintenance of environmental familiarity.

We observed no differential behavioral reactions towards the scents of familiar and strange conspecifics. Using training techniques, Rasa (1973) in the African dwarf mongoose, Kruuk et al. (1984) in the badger, and Clapperton et al. (1988) in the ferret have shown that the animals could discriminate scents from different individuals and groups of individuals. It seems possible that by using such methods, this ability could also be demonstrated in martens. Nevertheless, spontaneous behavioral reactions towards scent marks did not reveal such an ability. It would also be interesting to see whether martens would discriminate between fecal pellets from familiar and strange conspecifics. Along the same lines, chemical analysis of glandular compounds from the abdominal

TABLE 2. MEAN DURATION OF SNIFFING AND MEAN MARKING FREQUENCIES (TOTAL/NUMBER OF 5-MIN PERIODS) IN FEMALES A AND B ON OBJECTS CARRYING VARIOUS ODORS

Conditions	Female A		Female B	
	Mean duration of sniffing (sec)	Mean frequency of marking	Mean duration of sniffing (sec)	Mean frequency of marking
Unscented	9.64 ± 4.46 [a]	1.1 ± 0.7 [a]	14.81 ± 10.56 [a]	1 ± 0.9 [a]
Animal's own mark	4.36 ± 4.16 [a]	0 [a]	7.18 ± 4.46 [a]	0.2 ± 0.4 [a]
Female marten's mark	7.94 ± 4.61	0.5 ± 0.5	12.18 ± 10.72	0.3 ± 0.4
Stone marten's mark	14.71 ± 13.28	0.9 ± 1.04	14.02 ± 9.98	1 ± 1.2
Genet's mark	14.63 ± 15.59	0.8 ± 0.97	13.48 ± 10.47	1.3 ± 1.6

[a] $P < 0.05$.

glands might show individual variations as in anal sac secretions of otters (*Lutra lutra*) (Gorman et al., 1978), anal sac compounds of stoats (*Mustela erminea*) (Erlinge et al., 1982), and subcaudal glands of badgers (*Meles meles*) (Kruuk et al., 1984).

Data on sex recognition in mustelids are scarce (e.g., Crump, 1980; Clapperton et al., 1988, in the ferret). In our study, no significant results were obtained (experiment 1), but these negative findings do not necessarily imply that no information about sex is transmitted by olfactory cues. Seasonal variations in marking activity (De Monte and Roeder, 1990) suggest that olfactory cues are involved in reproduction. It is conceivable that outside the rutting period, scent marks from animals of both sexes could have the same meaning for males and females and simply indicate the presence of a stranger. In mustelids, social organization is generally considered to be based on intrasexual exclusion (Powell, 1979). In martens in particular, spatial distribution is not strictly restricted to such an organization but includes overlapping of home ranges among neighboring males and females (Pulliainen, 1982). This could explain why our animals reacted in the same way to the presence of male and female scent marks. It would be interesting to know how olfactory cues (especially scent marks) act during the rutting period and whether they convey information about the physiological state of females.

In experiment 2, there was no evidence of interspecific discrimination. Reactions towards scent marks of stone martens (a phylogenetically closely related species partly sympatric with the pine marten) and genets did not differ from those towards control objects or the martens' own marks. These results provide no evidence that olfactory cues are involved in interspecific relations between these two sympatric species.

In conclusion, the study of spontaneous responses of martens towards various scent marks gave no evidence for intra- or interspecific recognition. The only condition in which duration of sniffing *and* marking frequency were modified was in the presence of the animal's own scent. These results suggest that scent marking in martens may be a self-oriented behavior and act in "autocommunication" (Roeder, 1985). It remains to be clarified whether other functions ascribed to scent marking (territorial maintenance, individual recognition, pair-bond formation; see Thiessen and Rice, 1976; Gosling, 1982) should be considered as primary functions in this species or as outcomes of scent marking used for familiarization (Kleiman, 1966). Further behavioral studies (e.g., on marking activity during the rutting period and during social encounters) are necessary for clarifying the functions of scent marking in martens.

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SOLDIER DEFENSE SECRETIONS OF MALAYSIAN FREE-RANGING TERMITE OF THE GENUS *Lacessititermes* (ISOPTERA, NASUTITERMITINAE)

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Abstract—Soldiers of free-ranging termites of the genus *Lacessititermes* (Isoptera, Nasutitermitinae) secrete from their frontal glands a mixture of monoterpenes, sesquiterpenes, and diterpenes. *Lacessititermes ransoneti*, *L. laborator*, and *L.* species A produce species-specific secretions, the composition being most complex for *L. laborator*. Apart from known mono- and dihydroxytrinervitadienes, the following new diterpenes were isolated and tentatively assigned as trinervita-1(15),8(19)-dien-2β,3α,9α,14α-tetraol 2,3,14-*O*-triacetate, trinervita-1(15),8(19)-dien-2β,3α,9β,14α-tetraol 2,3,14-*O*-triacetate, 2β,3α,9α,14α-tetraacetoxy-1(15),8(19)-trinervitadiene, and 2β,3α,11α,13α-tetraacetoxy-1(15),8(19)-trinervitadiene. Data on intragenus chemical variations were subjected to canonical discriminant analysis and genetic distances among the species were calculated to depict intragenus identities and affinities.

Key Words—Isoptera, Termitidae, defense secretions, interspecific variations, *Lacessititermes*, diterpenes.

INTRODUCTION

Diterpenes, together with monoterpenes and less frequently sesquiterpenes, form the base chemicals of the defense secretion of termite soldiers of the subfamily Nasutitermitinae. This subfamily, of which over 500 species have been reported (Tho, 1982), contributes the largest number of species within a subfamily. The

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evolution of defense chemicals in the soldier termites has contributed, to a large extent, to successful defense of their colonies against predators. Studies on the chemical constitution of soldier termite secretions in relation to their phylogeny have indicated that the regression of their mandibles occurs concurrently with the development of chemicals (Goh et al., 1984; Prestwich, 1986). In the monophyletic scheme of the evolution of nasute termites, diterpene biogenesis developed somewhere along the evolutionary line close to *Longipeditermes* and *Hirtitermes* (Goh et al., 1984). The secretions of *Longipeditermes longipes* [morphologically identified as a single species (Tho, 1982)] contain diverse diterpene structural types from which the existence of three chemotypes within the species could be deduced. Similarly, based on diterpene chemistry from the relatively more advanced genus *Hospitalitermes*, two chemotypes were discovered from a single species of *Hospitalitermes umbrinus* (Chuah et al., 1983, 1986, 1987; Goh et al., 1988).

The genus *Lacessititermes* is restricted to the Indomalayan region with 15 known species. Although eight species have been recorded from Malaysia (Tho, 1982), only *L. ransoneti* and *L. laborator* are commonly encountered. The termites are characterized by having long antennal segments, long legs with the hind legs much longer than the body length, and highly pigmented body colors. Behaviorally, all species recorded have free-ranging habits; they march out in long columns without covered trails. The columns, starting from arboreal nests built around small branches of trees, often extend to more than 20 m. Food comprises scrapings off the surfaces of decomposing leaf litter, twigs, and bark of trees. They often feed on lichen growths on the tree. The food material is gathered and rolled into a small ball by the worker caste and is then carried all the way back, along the trail, to the nest. The soldiers can be monomorphic and dimorphic, and they protect their foraging columns of workers by positioning themselves along the fringes of the columns. Defense is effected by ejecting chemical secretions at their enemies. Our field observations so far have indicated that these termites, while on the march, are seldom attacked by predators, which would presumably attest to the effectiveness of their defense strategy. Additionally, the relatively long legs of the soldier termites of this genus are well adapted to enable them to move swiftly to converge at the sites of disturbances.

METHODS AND MATERIALS

Termite Material. Soldier termite materials used for the present studies were all collected from within closed canopy dipterocarp forests from the following localities in Peninsular Malaysia (Figure 1): (1) Muka Head Field Station [5°20′N, 100°8′E; 200 m above sea level (ASL)]—a coastal hill dipterocarp

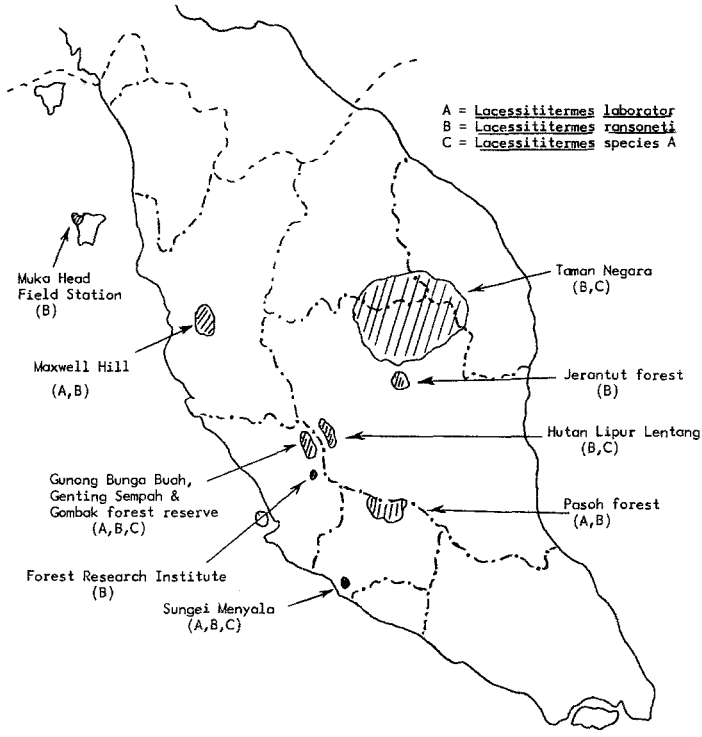
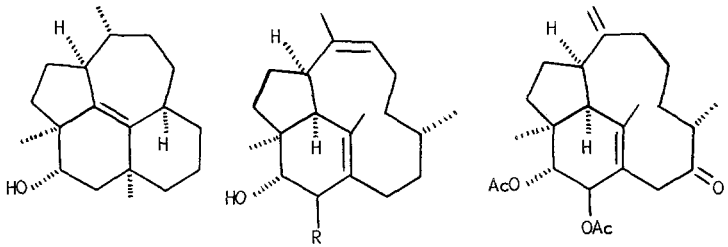


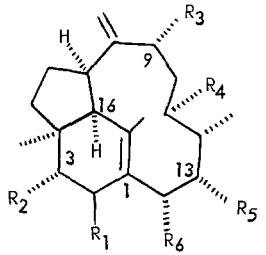
FIG. 1. Distribution of *Lacessititermes* in Peninsular Malaysia.

forest reserve in the state of Penang; (2) Maxwell Hill forest reserve (4°50′N, 100°50′E; 1000 m ASL)—an upper hill dipterocarp forest in the state of Perak; (3) Taman Negara (4°20′N, 102°25′E; 1000 m ASL)—the national park of Peninsular Malaysia in the state of Pahang; (4) Jerantut forest reserve (3°50′N; 102°20′E; 50 m ASL)—a forest reserve area in the state of Pahang; (5) Hutan Lipur Lentang forest reserve (3°30′N, 100°30′E; 300 m ASL)—a hill dipterocarp forest in the state of Pahang; (6A) Gombak forest reserve (3°20′N, 101°46′E; 300 m ASL), (6B) Gunong Bunga Buah forest reserve (3°10′N, 101°46′N; 600 m ASL), and (6C) Genting Sempah forest reserve (3°14′N, 101°46′E; 500 m ASL)—6A–6C are all hill dipterocarp forests in the state of Selangor; (7) Forest Research Institute (3°10′N, 101°30′N; 50 m ASL)—a forest research station at Kepong in the state of Selangor; (8) Pasoh forest reserve (2°58′N, 101°55′E; 100 m ASL)—a lowland dipterocarp forest in the state of Negeri Sembilan; and (9) Sungei Menyala forest reserve (2°29′N, 101°55′E; 100 m ASL)—a lowland dipterocarp forest in the state of Negeri Sembilan.

Analytical Methods. Gas chromatography (GC) and spectral data were recorded as described previously (Chuah et al., 1983, 1986, 1989; Goh et al., 1984, 1988). Monoterpenes (I–XII) and diterpenes (XIII–XXIX) as shown in Figure 2 were characterized by NMR and MS spectral data by comparison to reported data (Braekman et al., 1984, 1986; Dupont et al., 1981; Prestwich, 1979; Valterova et al., 1986; Vrkoč et al., 1978) and by coinjection with identical compounds previously characterized (Chuah et al., 1983, 1986, 1989; Goh et al., 1984, 1988). The secretion of *L. laborator* was fairly complex and repeated HPLC (SiO_2) chromatographic cycles were performed to obtain compounds XXIV–XXIX. The diterpenes isolated by HPLC were checked for purity by GC and TLC on Merck 5 × 10-cm 0.25-mm silica gel 60 plates. Four new diterpenes (XXIV, XXV, XXVI, and XXIX) were identified and tentative structures were assigned based on [^{1}H]NMR and MS data. [^{1}H]NMR data are given in Table 1.

Statistical Analysis. The relative mean proportions of monoterpenes and diterpenes were calculated. Differences between species were estimated by the





XIV R_1=R_3=R_4=R_5=R_6=H, R_2=OH
XVI R_1&(1-H)=O, R_2=OH, R_3=R_4=R_5=R_6=H
XVII R_1=OAc, R_2=OH, R_3=R_4=R_5=R_6=H
XVIII R_1=R_2=OH, R_3=R_4=R_5=R_6=H
XX R_1=R_2=OH, R_3=OAc, R_4=R_5=R_6=H
XXI R_1=R_2=OAc, R_3=OH, R_4=R_5=R_6=H
XXIII R_1=R_2=R_3=OAc, R_4=R_5=R_6=H
XXIV R_1=R_2=R_6=OAc, R_3=OH, R_4=R_5=H
XXV R_1=R_2=R_6=OAc, R_3=OH(β), R_4=R_5=H
XXVI R_1=R_2=R_3=R_6=OAc, R_4=R_5=H
XXVII R_1=R_2=R_3=R_4=OAc, R_5=R_6=H
XXVIII R_1=R_2=R_3=R_5=OAc, R_4=R_6=H
XXIX R_1=R_2=R_4=R_5=OAc, R_3=R_6=H

FIG. 2. Diterpenes from the defense secretions of *Lacessititermes*.

TABLE 1. COMPARISON OF [^{1}H] NMR (100 MHz)[a] SPECTRA ($CDCL_3$) OF DITERPENES FROM *Lacessititermes laborator*

Proton	XXIV	XXV	XXVI	XXVII	XXVIII	XXIX
H-2	5.6brd (9)	5.6brd (9)	5.6brd (8)	5.6brd (9)	5.6brd (8)	5.8brd (8)
H-3	5.5brd (9)	5.5brd (9)	5.56brd (8)	5.5brd (9)	5.4brd (8)	5.7brd (8)
H-7	3.1ddd (11,11,7)	3.0ddd (10,10,7)	3.1ddd (10,10,6)	3.2ddd (11,11,9)	3.2ddd (10,10,6)	3.0ddd (10,10,8)
H-9	4.4dd (9,2)	4.5dd (9,2)	5.3dd (9,2)	5.3dd (9,2)	5.3dd (9,2)	
H-11				4.6m		4.8m
H-13					5.0m	5.0m
H-14	4.8dd (6,3)	4.8dd (6,3)	5.2dd (6,3)			
H-16	2.46d (11)	2.45d (11)	2.5d (11)	2.33d (11)	2.5d (11)	2.35d (11)
H_3-17	1.7brs	1.7brs	1.7brs	1.76brs	1.6brs	1.7brs
H_3-18	1.05s	1.00s	0.98s	0.98s	1.05s	0.97s
H_2-19	5.1brs 5.2brs	4.9brs 4.93brs	5.2brs 5.3brs	5.30brs 5.50brs	5.4brs 5.5brs	5.2brs 5.1brs
H_3-20	0.98d (6.5)	0.90d (6.5)	0.89d (6.5)	0.97d (6.5)	0.93d (6.5)	0.90d (6.5)
$OCOCH_3$	2.03 2.05 2.06	2.03 2.04 2.13	2.01 2.03 2.04 2.05	2.00 2.04 2.07 2.26	2.01 2.03 2.04 2.13	2.01 2.04 2.05 2.06

[a] 100 MHz. br, s, d, t, and m = broad, singlet, doublet, triplet, and multiplet, coupling constant (Hz) in parenthesis.

Kruskal-Wallis test (Table 2). Linear discriminant analysis was performed on the data, with species as the independent variable and mono- and diterpenes separately as dependent variables (Figure 3). Dependent variables were entered simultaneously into the discriminant function. The following discriminant function statistics were computed: (1) Discriminant scores and from which all group scatterplots were drawn. An estimation of the population of each species correctly classified to mono- and diterpene compositions was made. (2) Wilks'

TABLE 2. MEAN AND STANDARD ERROR (SE) FOR *Lacessititermes*

Compound	*L. ransoneti*[a] (mean ± SE)		*L. laborator*[b] (mean ± SE)		*L. sp A*[c] (mean ± SE)		P^d
Monoterpenes							
α-Pinene (I)	33.36	5.783	59.29	1.604	56.00	2.449	<0.001
Camphene (II)	1.18	0.405	1.43	0.535	1.50	0.577	ns
β-Pinene (III)	11.64	1.502	8.14	3.288	9.75	1.708	<0.05
Myrcene (IV)	1.36	0.674	1.71	0.756	1.25	0.500	ns
α-Phellandrene (V)	1.55	0.934	1.14	0.378	0.00	0.000	<0.01
Δ^3-Carene (VI)	11.64	1.936	3.71	1.604	2.50	0.577	<0.001
α-Terpinene (VII)	1.18	0.405	2.14	1.215	0.00	0.000	<0.001
Limonene (VIII)	28.82	2.183	16.71	1.380	21.50	1.291	<0.001
β-Phellandrene (IX)	2.09	1.758	1.71	1.254	1.25	0.500	ns
p-Cymene (X)	1.73	0.786	0.00	0.000	0.00	0.000	<0.001
γ-Terpinene (XI)	1.09	0.302	0.00	0.000	1.50	0.577	<0.001
Terpinolene (XII)	4.36	1.567	3.71	1.890	4.75	0.957	ns
Diterpenes							
XIII	4.64	2.541	1.43	0.535	2.75	0.500	<0.01
XIV	5.09	1.300	0.00	0.000	0.00	0.000	<0.001
XV	3.45	2.770	2.57	0.535	1.25	0.500	ns
XVI	9.45	2.841	2.00	0.817	6.50	1.291	<0.001
XVII	6.00	2.098	3.14	0.378	6.25	0.957	<0.01
XVIII	38.36	4.154	5.14	0.900	52.00	1.414	<0.001
XIX	6.09	3.780	1.86	0.900	8.75	1.258	<0.01
XX	17.73	3.438	2.29	0.951	7.25	0.957	<0.001
XXI	5.27	1.954	5.14	0.690	7.00	0.817	ns
XXII	1.82	0.874	3.29	0.756	3.25	0.957	<0.01
XXIII	2.09	1.375	5.43	1.718	5.00	0.817	<0.01
XXIV	0.00	0.000	4.71	1.113	0.00	0.000	<0.001
XXV	0.00	0.000	8.29	1.113	0.00	0.000	<0.001
XXVI	0.00	0.000	7.28	1.113	0.00	0.000	<0.001
XXVII	0.00	0.000	3.86	0.900	0.00	0.000	<0.001
XXVIII	0.00	0.000	25.71	1.704	0.00	0.000	<0.001
XXIX	0.00	0.000	17.86	0.900	0.00	0.000	<0.001

[a] Range for 12 colonies.
[b] Range for 7 colonies.
[c] Range for 5 colonies.
[d] Kruskal-Wallis test: ns = nonsignificant at the 0.05 level.

lambda, which tests the null hypothesis that species means are equal. (3) Standardized canonical discriminant function coefficients, to identify the most important dependent variables for discriminating among species. All analyses were run on the SPSS/PC$^+$ program (Norusis, 1986).

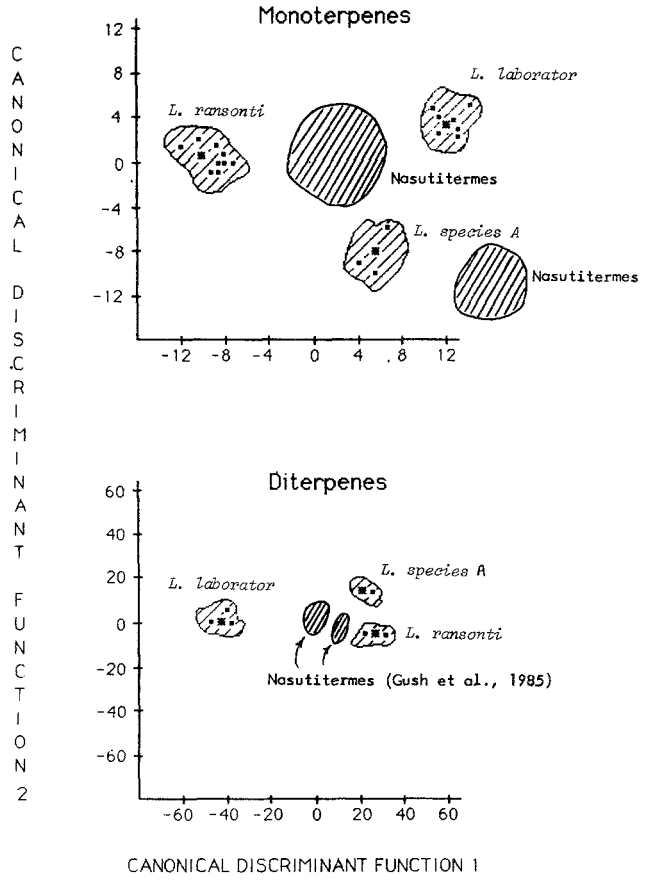


FIG. 3. Canonical discriminant plots for *Lacessititermes*.

The genetic distance between two species was calculated using the mean relative proportions of all the mono- and diterpene compounds following the formula of Nei (1972):

$$D_{XY} = -\ln\left[\frac{\sum X_i Y_i}{\sqrt{\left[\sum X_i^2 \sum Y_i^2\right]}}\right]$$

where X and Y are the mean relative proportion of compound i in species X and Y, respectively. A dendrogram using the unweighted pair-group arithmetic average clustering method was constructed (Figure 4).

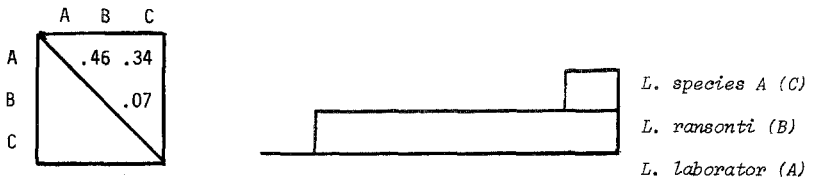


FIG. 4. Matrix and dendograms constructed from genetic distances among *Lacessititermes*.

RESULTS AND DISCUSSION

The genus *Lacessititermes* is closely related to the previously studied *Hospitalitermes* (Chuah et al., 1983, 1986, 1987). The three species of *Lacessititermes* presently studied show the same tricyclic diterpene (trinervitene) skeletal type that is dominant in their diterpene secretions. Based on the chemical compositions, only one chemotype was evident for each of the three species studied from colonies collected over many locations in Malaysia (Figure 1). As with many other genera, the convergence towards the trinervitane tricyclic skeleton may be a feature associated with the more advanced nasute soldier and distinct levels of evolution showing this trend have been represented by a monophyletic regression line (Goh et al., 1984). Variations in chemical composition of the defense secretions in the Nasutitermitinae would have been the result of selective natural forces that impact upon the various species in their adaptation to the environment. This would include predation pressure, behavioral changes as a response to food preferences, and reproductive isolation through a need for interspecific identities. Hence, it has been suggested (Prestwich, 1984) that compositional and structural variations in the defense chemicals may be viewed as an indirect way of measuring the genetic variation within a given taxon and may be used as chemosystematic criteria for identifying species in morphologically ambiguous cases.

Limonene, α-pinene, and β-pinene are noted as the most abundant monoterpenes in the defense secretions of *Lacessititermes* soldiers. These monoterpene hydrocarbons, being natural repellents, are common in the secretions of all the three species. As many as 12 monoterpenes (I–XII) are recorded for *L. ransoneti*, indicating a "broad spectrum" pattern (Table 2) in contrast to a greater preference for selected monoterpenes, as shown by non-free-ranging *Nasutitermes* termites (Chuah et al., 1989). Each soldier termite of *L. ransoneti* provides defense secretion of 1.0–3.3% of its body weight consisting of 4.7–7.2 μg monoterpenes, 0.5–1.5 μg sesquiterpenes, and 7.7–11 μg diterpenes. Similarly each *L. laborator* soldier gives 2.4–3.2% of secretion consisting of 5.3–7.7 μg monoterpenes, 12–21μg sesquiterpenes, and 31–41 μg diterpenes.

For *L.* species A the value is 2.0–2.3% of secretion consisting of 7.2–8.6 μg monoterpenes, 1.5–2.9 μg sesquiterpenes, and 13.6–15.2 μg diterpenes. Thus, only *L. laborator* secretes a relatively large proportion of sesquiterpenes in comparison to the other terpenes. GC-MS spectral data show that the sesquiterpenes are hydrocarbons and alcohols. A few of the larger components are tentatively assigned as β-caryophyllene (55%), longifolene (10%), α-gurjunene (20%), and γ-gurjunene (15%) based on their EI-MS fragmentation spectral data and by coinjection with commercially available standards. Among the species studied, variations in the composition of the monoterpenes, sesquiterpenes, and diterpenes can be used to provide diagnostic patterns that depict intergeneric and interspecific differences.

As shown by the present study, the defense secretions of these free-ranging termite soldiers appear to be characterized by having a relatively wide spectrum of volatile chemical components (Table 2) in comparison to more selective terpenoids found for the covered-trail termite *Nasutitermes* (Chuah et al., 1989). This could be associated with their free-ranging habits and behavioral adaptations; their free-ranging columns extend over considerable distances and are unprotected by any sheltered cover. Thus a wide spectrum of volatile monoterpene components is needed to repel a larger number of arthropods.

The defense secretion of *L. laborator* is especially noteworthy as being relatively complex (Table 2) because as many as 17 diterpene components can be detected by GC and isolated by HPLC. Eleven of the diterpenes (XIII–XXIII), also present in the defense secretions of *L. ransoneti*, are known from previous studies on *Longipeditermes longipes* (Goh et al., 1984), *Hospitalitermes* species (Chuah et al., 1986), and *Nasutitermes* species (Chuah et al., 1989).

Diterpenes XXVI, XXVII, XXVIII, and XXIX all had $M^{+\cdot}$ at 504, compatible with the molecular formula $C_{28}H_{40}O_8$, and characteristic fragment ions of *m/z* 444, 384, 324, and 264 corresponding to the elimination of one to four molecules of acetic acid and strong ions of *m/z* 60 and 43 ($AcOH^{+\cdot}$ and CH_3CO^+, respectively) were recorded. As the MS of diterpenes XXVI and XXIX are almost identical not only to each other but also to those of tetraacetates (XXVII and XXVIII) previously isolated from *Nasutitermes* species (Braekman et al., 1984), they are assigned as 1(15),8(19)-trinervitadiene derivatives bearing four acetoxy groups. The [^{1}H]NMR spectral data are in agreement with this hypothesis since four ^{1}H signals, attributable to protons on carbon-bearing acetoxyl groups, appear in the low field region (see Table 1). The [^{1}H]NMR spectral data of XXVI and XXIX are quite similar to those reported for 2β,3α,9α,13α-tetraacetoxy-1(15),8(19)-trinervitadiene (XXVIII) from *Nasutitermes PNG (F)* and 2β,3α,9α,11α-tetraacetoxy-1(15),8(19)-trinervitadiene (XXVII) from *Nasutitermes gracilirostris* reported by Braekman (1984).

A comparison of the proton chemical shifts of the diterpene XXVI to those of the reported diterpene XXVIII reveals some similarities except that the absorption of δ5.2 (H-14) is a doublet of doublet (6 and 3 Hz) for XXVI instead of δ5.0 (H-13, multiplet) for XXVIII. Thus, the diterpene XXVI is tentatively assigned as 2β,3α,9α,14α-tetraacetoxy-1(15),8(19)-trinervitadiene.

The chemical shifts of XXIX for protons H-2, H-3, H-7, H-13, H-16, H_3-17, H_3-18, H_2-19, H_3-20 and acetyl groups match quite closely with those of the reported diterpene XXVIII. Diterpene XXIX's absorption at δ5.00 (multiplet) is assigned to H-13 due to the similarity of the splitting pattern of this proton to that in the known 2β,3α,9α,13α-tetraacetoxy compound XXVIII. The chemical shift of δ4.8 (multiplet) is assigned for proton H-11 to an acetoxy group, in close agreement to that reported for XXVII by Braekman et al. (1984). Based on this data, the diterpene XXIX is tentatively assigned as 2β,3α,11α,13α-tetraacetoxy-1(15),8(19)-trinervitadiene (XXIX).

The mass spectra of triacetyl tetraols XXIV and XXV, with $M^{+\cdot}$ (462) compatible with the molecular formula $C_{26}H_{38}O_7$ and characteristic fragment ions of *m/z* 402, 342, and 282 corresponding to the elimination of one to three molecules of acetic acid as well as elimination of ketenes, are almost identical not only to each other but also to that of trinervita-1(15),8(19)-dien-2β,3α,14α-triol 2,3,14-*O*-triacetate reported by Braekman et al. (1984). The fragmentation pattern is suggestive of a 1(15),8(19)-trinervitadiene skeleton bearing three acetoxyl groups and a hydroxy group (indicated by the elimination of H_2O). The [^{1}H]NMR (100 MHz) of compound XXIV also provides evidence for the presence of three acetoxy groups (δ = 2.03s, 2.05s, and 2.06s) of which two assume the positions 2β and 3α [doublets at δ = 5.5 (9 Hz) and 5.6 (9 Hz)] and the third at position 14α [δ = 4.8dd (6 and 3 Hz)]. The hydroxyl group is assigned at carbon 9 based on the doublet of doublet at δ4.4 (9 and 2 Hz). Thus the structure is tentatively assigned as trinervita-1(15),8(19)-dien-2β,3α,9α,14α-tetraol 2,3,14-*O*-triacetate (XXIV). Diterpene, XXV, possessing an identical MS spectrum as that of XXIV, is an H-9 positional epimer of XXIV. The proton chemical shift at δ4.5 (doublet-doublet of 9 and 2 Hz) can be assigned to the 9β configuration because the exomethylene protons are shifted to higher field (δ = 4.9 and 4.93 compared to δ5.1 and δ5.2 for XXIV). The diterpene XXV is thus tentatively assigned as trinervita-1(15),8(19)-dien-2β,3α,9β,14α-tetraol, 2,3,14-*O*-triacetate (XXV).

The data obtained from soldier defense secretions of *L. laborator*, *L. ransoneti*, and *L.* species A provide an opportunity for further statistical analysis, particularly canonical discriminant analysis and calculation of genetic distances. The Kruskal-Wallis tests indicate greater variation in diterpenes than in the monoterpene distribution among species (Table 2). Of a total of 12 monoterpenes (I–XII) and 17 diterpenes (XIII–XXIX), four components from monoterpenes (camphene, myrcene, β-phellandrene, and terpinolene) and two

from diterpenes (XV and XXI), respectively, were not significantly different (Kruskal-Wallis test, $P > 0.05$) among the species (Table 2). Apart from the most abundant monoterpenes, α-pinene, β-pinene, and limonene, which were similar for all three species investigated, monoterpene compositional patterns were readily distinguishable. The distribution of diterpenes as described earlier was distinct (Table 2) for *L. laborator* compared to the two other species. Diterpenes (XXIV–XXIX) were found to be present only in *L. laborator*, while diterpenes XVIII and XX were found to be present in relatively small amounts in comparison to the other two species.

Discriminant analysis of the monoterpenes and diterpenes gave significant values for Wilks lamda ($P < 0.001$) for both the first and second discriminant functions, the former accounting for 92.6% and 99.7% of the total variance for monoterpene and diterpene, respectively. Species could then be differentiated by the distribution of these compounds. A comparison of the standardized discriminant function coefficients showed that the three most important compounds for discriminating among species were α-pinene (I), Δ^3-carene (VI), and limonene (VIII) for the monoterpene content and trinervita-1(15),8(19)-dien-2β,3α-diol (XVIII), trinervita-1(15),8(19)-dien-2β,3α,9α,14α-tetraol 2,3,14-*O*-triacetate (XXIV) and 2β,3α,9α,14α-tetraacetoxy-1(15),8(19)-trinervitadiene (XXVI) for the diterpene content. As such, species were correctly classified (100%) according to monoterpene or diterpene compositions for *L. ransoneti*, *L. laborator* and *L.* species A. This is graphically expressed by the scatterplots for the two discriminant function axes (Figure 3). This is in contrast to that reported by Gush et al. (1985) for four species of *Nasutitermes*, in which correct classification was 98.9% and 76.1%, respectively, for monoterpene and diterpene as discriminating variables (also included in Figure 3).

With the assumption that the defense chemicals are genetic in nature, the genetic distances between the species are computed, and the results are as follows: *L.* species A and *L. laborator*: 0.34, *L.* species A and *L. ransoneti*: 0.07, and *L. ransoneti* and *L. laborator*: 0.46. The dendrogram constructed from these values is shown in Figure 4. The results show that *L. laborator* is distinct from *L.* species A and *L. ransoneti* but having a closer affinity with the latter.

Discriminant analysis has shown conclusively that both monoterpenes and diterpenes can be used to distinguish among these species of the genus *Lacessititermes* used in this study (Figure 3). The discriminant functions were significant and very accurate (100%) in assignment ability for both types of terpenoids. Although the present study provided little ambiguity because of the definite chemical patterns of the secretions among the three species, further data on other species and genera may provide more challenging problems for such statistical analysis. Earlier results with monoterpenes and diterpenes as dependent variables reported for the genus *Nasutitermes* by Gush et al. (1985) were also indicative of the usefulness of such analysis.

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ROBBER BEES (*Lestrimelitta limao*) AND THEIR HOST Chemical And Visual Cues In Nest Defense By *Trigona* (*Tetragonisca*) *angustula* (Apidae: Meliponinae)

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Abstract—The nest of the stingless bee, *Trigona (Tetragonisca) angustula*, is guarded by bees positioned in the nest entrance and others hovering in front of it. Hovering guard bees track returning foragers sideways along the last 10 cm in front of the nest, but intercept and incapacitate nest intruders by clinging with mandibles to wings and legs. When attacked by the cleptobiotic stingless bee *Lestrimelitta limao*, the colony strengthens its aerial defense with hundreds of additional hoverers. To test our hypothesis that this reaction is due to interspecific chemical communication based on kairomone effects, we presented synthetic cephalic volatiles of both species at the nest entrance and counted the number of bees leaving the nest and taking up hovering positions. We conclude that guard bees recognize *L. limao* by the major terpenoids of their volatile cephalic secretions, geranial, neral (=citral) and 6-methyl-5-hepten-2-one; other components may fine-tune this recognition. The effect of chemical stimuli is not significantly enhanced by combination with a dummy of *L. limao*. Guard bees, we hypothesize, respond to this kairomone by secreting a species specific alarm pheromone; a major component of this pheromone, benzaldehyde, recruits additional bees to defend the nest.

Key Words—Cephalic volatiles, kairomone, nest defense, *Lestrimelitta limao*, *Trigona (Tetragonisca) angustula*, stingless bees, Hymenoptera, Meliponini, Apidae.

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INTRODUCTION

In species of stingless bees, especially those that have nest entrance tubes with wide openings, disturbances in the periphery of the nest evoke rapid attacks by outflying guard bees, often followed by recruitment of additional defending bees (Wille and Michener, 1973; Michener, 1974). Generally, these mass attacks are triggered by alarm pheromones. Other species of stingless bees, especially those that have small nest entrances with diameters of one or a few bees' heads, rarely react aggressively to disturbances of their nest. Instead, they vacate or close the entrance tube before retreating into the nest. *T. angustula* (formerly *Trigona jati*) responds to general disturbance around the nest by retreating. However, they possess a nest defense that may be unique among stingless bees: Besides guard bees occupying the nest entrance tube, there are additional guards that hover in stable positions on both sides in front of the nest entrance and its close vicinity (Wittmann, 1985; Zeil and Wittmann, 1989). These hovering guards attack flying insect intruders and pull them to the ground by clinging to their wings or legs.

The value of this defensive behavior becomes obvious when attacks of the cleptobiotic stingless bee, *Lestrimelitta limao*, are taken into account. Bees of this genus do not visit flowers, but instead rob nests of other stingless bees of nectar, pollen, and nest construction material. Scout bees, after having detected a host colony, recruit hundreds of nestmates for a raid.

T. angustula workers defend their nests against attacks of *L. limao* by intercepting a scout in front of the nest or by mass recruitment of additional hovering guard bees. We and others (Sakagami et al., 1989) have observed these bees successfully fighting off even full-scale attacks of the robber bees. Earlier experiments on the defensive behavior suggested that the recruitment of additional hovering guards was triggered by a kairomone secreted by *L. limao* workers (Wittmann, 1985).

The major components of mandibular gland secretions in *L. limao* are the monoterpenes neral and geranial (= citral; Blum, 1966). Our recent gas chromatographic and mass spectrometric analysis of cephalic volatiles revealed additional compounds. Here we report on studies that identify the chemical and visual cues involved in the recognition of intruders and in the recruitment of hovering guard bees during raids of *L. limao*.

METHODS AND MATERIALS

Heads of *Lestrimelitta limao* workers that participated in raids were extracted in pentane. Gas chromatographic and mass spectroscopic analyses were carried out on a Varian MAT 311 A coupling system using a 50-m ×

TABLE 1. MAJOR CEPHALIC VOLATILES OF *Lestrimelitta limao* AND THEIR RELATIVE PROPORTIONS[a]

Compound	%
Hydrocarbons	
Heneicosane	0.9
(Z)-9-Heneicosene	2.5
Tricosane	0.2
(Z)-9-Tricosene	10.0*
Pentacosane	0.9
Alcohols	
Hexadecanol	0.2*
(Z)-7-Hexadecenol	2.5*
(Z)-9-Octadecenol	2.0*
Acetates	
(Z)-7-Hexadecenyl acetate	2.0
(Z)-9-Octadecenyl acetate	2.0
Ethyl esters	
Ethyl tetradecanoate	0.2
Ethyl hexadecanoate	0.2
Ethyl octadecanoate	0.2
Ethyl(Z)-9, Z-12-octadecadienoate	0.2
Ketones	
6-Methyl-5-hepten-2-one	1.0*
Aldehydes	
Neral/geranial (citral)	75.0*

[a] Single compounds tested are marked with an asterisk, compound mixtures tested are indicated with brackets.

0.25-mm-ID fused silica column with FFAP-CB as a stationary phase. Confirmation of the structures of identified compounds is based on comparison of mass spectra of gas chromatographic retention times with pure synthetic reference samples. Pure synthetic samples and mixtures of the identified compounds (Table 1) were tested in a bioassay designed to evaluate their properties in interspecific chemical communication.

From 1984 until 1987 one nest of *L. limao* and two of *T. angustula* were kept under observation in a suburbian area of Porto Alegre, Rio Grande do Sul, in southern Brazil. In front of *T. angustula* nests the flight behavior of hovering guard bees and their response to returning foragers were filmed and reconstructed by single-frame analysis. We simulated approaches of flying insect intruders towards both nests by passing dried collection specimens as well as live and slightly squeezed workers of the robber bees through the group of hovering guards to the entrance tube. Mass attacks by robber bees were simu-

lated by presenting 1 μl (corresponding to 4–100 robber bees) of single volatile components, component mixtures, and a whole synthetic pheromonal bouquet (artificial head) of *L. limao* for 5 min to the guard bees at the nest entrance (Table 1). The volatiles were pipetted on cell bottoms punched from *Apis*-comb foundations and fixed on a 25-cm-long plastic stick slightly underneath the opening of the entrance tube. For each trial a new wax disk and a new stick were used. After testing, the stimulus was completely removed. The substances were presented in random sequence, with five replicates per test at either one of the nests. Furthermore, the same compounds were presented together with a shiny black pin head serving as a dummy of the similarly sized and colored robber bees. The reaction of guard bees during stimulus presentation was quantified by counting the number of bees that left the nest and immediately took up hovering positions at its entrance. As controls, numbers of hovering bees were recorded for 5 min during pentane and during dummy presentation before each test. Trails were separated from each other by at least 30 min, in some cases by more than 3 hr, depending on the time a colony needed to reestablish control conditions.

Voucher specimens are deposited at the Fundação Zoobotânica do Rio Grande do Sul in Porto Alegre, Brazil and at the Zoological Institute of the University of Tübingen, West Germany.

RESULTS

Visual and Olfactory Discrimination of Intruders. Inspection of 16-mm films and video recordings revealed that hovering guard bees track incoming foragers on the final 10 cm of their return to the nest. These tracking episodes are not easy to see, since most of them only last about 100 msec and are difficult to distinguish from spontaneous jittering movements of guard bees (Zeil and Wittmann, 1989). Figure 1 shows two longer sequences with typical tracking movements of guard bees. As guard bees prefer hovering positions close to the nest entrance and the flight corridor, they might be able to recognize foragers visually by their size. At a distance of 2 cm from the flight corridor—as in the sequences in Figure 1—a forager subtends horizontally about 15° and vertically about 6° at the eye of a guard bee. Horizontal and vertical interommatidal angles in the frontal visual field in *T. angustula* workers are 2.5°–4.5° and 1.2° respectively, as determined with the aid of the pseudopupil. At 2 cm, therefore, a forager will be seen by about 25 ommatidia.

Discrimination between returning foragers and intruders must, in addition, involve olfactory cues: We rarely observed attacks by guard bees on dried collection specimens of *L. limao* workers that were moved towards the nest entrance, whereas live robber bees that were fixed to a stick and approached to

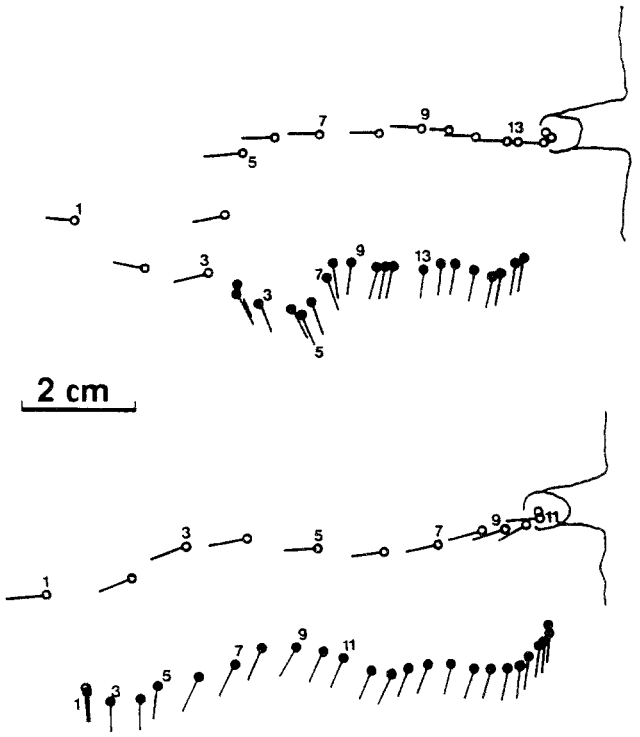

FIG. 1. Two sequences in which a guard bee tracks a returning forager seen from above. Circles mark the position of the head of guard bees (black) and foragers (open) every 20 msec, lines attached to circles represent the body axis orientation, positions are numbered consecutively. The nest entrance tube is shown at the right. Note that guard bees keep their orientation constant during tracking, except for a short turn in the direction of movement of the forager (between position 7 and 9, top sequence; and position 5 and 7 bottom sequence).

the nest were rapidly attacked by the guard bees (Wittmann, 1985). These attacks are fast (about 400 msec) and seem to be triggered whenever a robber bee passes through the frontal visual field of a guard bee (Figure 2). We did not observe guard bees turning towards dead or live robber bees when they were moved towards them from the side. From inspection of films, it is our impression that hovering guard bees tend to point into the wind during mass defense. Although we do not have a record of wind directions in front of the nest, we think that the following observations support the view that guard bees of *T. angustula* approach and attack a target against the wind: (1) consecutive attacks on a stationary live *L. limao* worker tend to occur from one direction only and

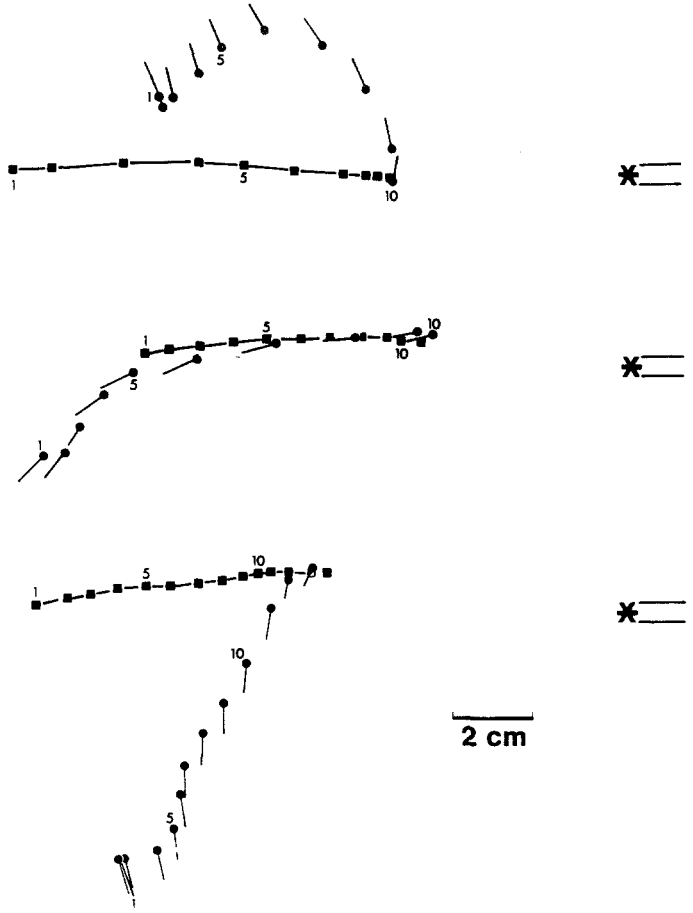

FIG. 2. Three attacks on a moving *L. limao* bee seen from above. Guard bee positions (black circles) and robber bee position (black squares) are shown every 40 msec; consecutive positions are numbered. The nest entrance tube is marked with an asterisk. At the end of each approach the bee has landed on the target. Note that the target is always seen in the frontal visual field of the attacking bee.

(2) the orientation of a bee is rather constant during the approach (Figure 3). We suggest, therefore, that an attack on a robber bee is most likely triggered by the coincidence of an olfactory perception and a target in the frontal visual field. However, since attacks are very fast, the actual execution of an attack must be under visual control.

When we presented cephalic volatiles of the robber bee to guard bees in

FIG. 3. Two approaches (left) and two attacks (right) on a stationary robber bee (square) recorded within 30 sec of each other. Conventions as before.

the nest entrance, slightly more workers were provoked to fly out when those chemical stimuli that had a recruiting effect were combined with a nonmoving black dummy. The effect, however, is not significant (t test, $P = 0.05$; Figure 4).

Interspecific Chemical Communication. Tests of identified compounds and mixtures of the cephalic volatiles of *L. limao* workers revealed that the alcohols, hydrocarbons, and esters did not elicit significant defensive response in *T. angustula* guards. In contrast, 6-methyl-5-hepten-2-one and citral provoked great numbers of *T. angustula* bees to fly out of the nest and take up hovering positions. Other bees fly in loops of about 1 m in diameter around the nest, thus patrolling its vicinity seeking out and attacking robber bees. The mixture of all compounds (artificial head of *L. limao*) elicited the strongest reaction among all synthetic volatiles tested. This response reached about 60% of that provoked

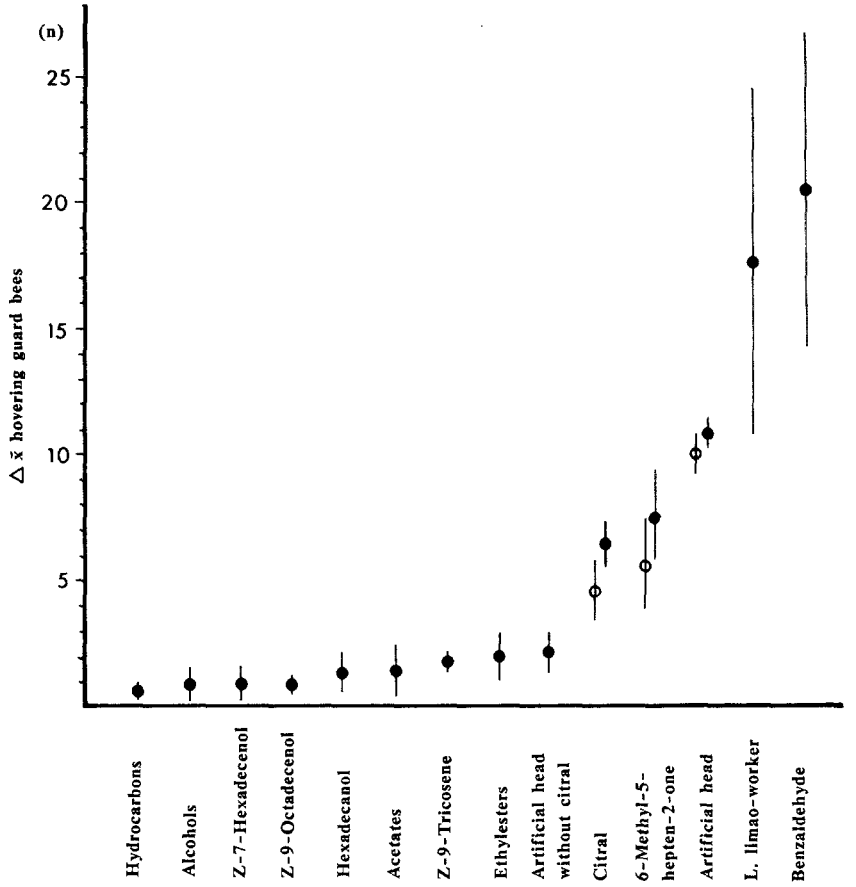

FIG. 4. Defensive reaction of *T. angustula* workers in response to single volatiles and compound mixtures presented 1 cm in front of the nest entrance tube without (circles) or together with a dummy (black dots). In five repetitions of each experiment, bees leaving the nest and taking up hovering positions were counted. Citral and 6-methyl-5-hepten-2-one released by the robber bees operate as the key substances in triggering the defense reaction (kairomone effect). Benzaldehyde is a component of the cephalic volatiles in *T. angustula* and a key substance of their alarm pheromone.

by a live robber bee (Figure 4). In none of the controls the number of hovering guard bees was increased.

These results demonstrate that cephalic volatiles of *L. limao* have a kairomonal effect on *T. angustula* workers, as they trigger their defense behavior. The question arises whether this kairomone operates as a direct releaser of the

massive defense reaction in *T. angustula* or whether its message is transformed by the guard bees into a species specific alarm signal.

Intraspecific Chemical Communication. In this context, it should be noted that *T. angustula* does not recruit additional hovering guard bees in response to general disturbances of the nest, like shaking, hitting, or blowing into the nest entrance. However, when 1 μl of benzaldehyde, one of the compounds of the mandibular gland secretion of *T. angustula* (Schröder and Francke, unpublished) is presented at the nest entrance, numerous bees are recruited to fly out and take up hovering positions. This reaction is as strong as the response to an alive *L. limao* (Figure 4).

DISCUSSION

The response to raids of the robber bees is not consistent within the Meliponinae: In some species of stingless bees, like *T. pectoralis*, *M. mexicana*, and *M. beecheii*, guard bees react aggressively when workers of *L. limao* are presented at the nest entrance (Weaver et al., 1975). On the other hand, colonies of a variety of species appear to be completely disoriented by the presence of citral (Moure et al., 1956), which in this case functions as an allomone (Blum et al., 1970). Guard bees and workers of other species retreat to the interior of the nest where they hide between brood combs, thus avoiding bee losses during pillages of *L. limao* (Nogueira-Neto, 1970).

Of the about 350 species of neotropical stingless bees, apparently only three (*Cephalotrigona capitata*, *T. angustula*, and *L. limao*) have a sympatric distribution that reaches from Mexico to southern Brazil (Camargo et al., 1988). During their long common history and due to high frequencies of aggressive encounters between *L. limao* and *T. angustula* (Schwarz, 1948; Wille, 1961; Nogueira-Neto, 1970; Sakagami et al., 1988) *T. angustula* has obviously adapted its nest guarding behavior and defensive strategy to raids of the robber bees.

The *T. angustula* colonies on which this study was carried out had not suffered any attacks of *L. limao* for more than six months before the experiments. Even if raids had been carried out in the absence of an observer, they could have easily been detected by high amounts of dead robber bees and nest defenders in front of the hives. As worker bees of *T. angustula* have a longevity of a few weeks, associative learning effects are not involved in their reaction to our simulated *L. limao* attacks.

Comparing the reactions of *T. angustula* to citral and 6-methyl-5-hepten-2-one with those to the more or less complete mixture of synthetic volatiles ("artificial head") suggests that those compounds which themselves cause no

or low responses in the guard bees, have a synergistic effect when added to these two volatiles.

The strengthening of the nest defense in *T. angustula* is, therefore, based on the recognition of *L. limao* workers by their pheromonal bouquet. Decisive for this are key volatiles (citral and 6-methyl-5-hepten-2-one), which are absent in the volatile secretions of the receiver (Schröder, 1985). In this basic feature, the interspecific communication of *L. limao* and *T. angustula* differs, for instance, from the reaction of workers of *T. fulviventris* on raids of the robber bees (Johnson, 1987). Here citral (geranial and neral), secreted by *L. limao*, is confused by the attacked bees with components of their own alarm pheromone (geraniol and nerol) and elicits their defense of the nest.

As already suggested by B. Smith (personal communication), benzaldehyde was found to be a major component of the alarm pheromone in *T. angustula*. However, these bees do not make use of their alarm pheromone to recruit additional guards during unspecific disturbances at the nest. Apparently, a necessary stimulus for its secretion is the presence of the kairomone released by *L. limao*.

We conclude that effective defense of the nest in *T. angustula* against pillages of the robber bees is achieved by two successive processes of inter- and intraspecific chemical communication: (1) The recognition of *L. limao* workers by their kairomone. This information, together with a visual stimulus, is sufficient to locally trigger attacks of the hovering guard bees on approaching robber bees. However, to strengthen the nest defense, even before a robber bee has made its way into the nest, the information on the attack has to reach the interior of the colony. (2) Apparently, the message of the kairomone is transformed by guard bees at the entrance into the species-specific alarm pheromone, which can be rapidly transmitted through the long inner entrance tube to recruit hundreds of additional nest mates that leave the nest and seek out targets for their aerial attacks.

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Guest Editorial

At the annual meeting of the International Society of Chemical Ecology in Göteborg, Sweden, August 7–11, 1989, the assembled membership unanimously adopted the principles embodied in the following resolution, drafted by a small group of us:

> Natural products constitute a treasury of immense value to humankind. The current alarming rate of species extinction is rapidly depleting this treasury, with potentially disastrous consequences. The International Society of Chemical Ecology urges that conservation measures be mounted worldwide to stem the tide of species extinction, and that vastly increased biorational studies be undertaken aimed at discovering new chemicals of use to medicine, agriculture and industry. These exploratory efforts should be pursued by a partnership of developing and developed nations in such fashion that the financial benefits flow in fair measure to all participants.

In its essence the resolution is self-explanatory. It expresses our concern for the loss of the very substances that are the focus of our professional interest, and it urges remedial action. The vast majority of natural products remains to be discovered. If, over time, we are to have the opportunity to prospect for these riches, biodiversity will need to be conserved. Species are now vanishing faster than they are being scrutinized chemically. To counter this alarming reality, we will need to accelerate the exploratory search for natural products, while at the same time expanding the efforts to conserve nature itself.

The question, of course, is how to proceed. Species loss is most pronounced in the tropics, where biodiversity is greatest, yet the nations are also the poorest and least able to implement conservation programs. What the resolution proposes is that a substantial fraction of the cash flow stemming from the commercialization of new natural products be specifically channeled to Third World nations, where these funds could in turn help foster the conservation effort. Developing nations are the repositories of much of nature's remaining undiscovered chemical wealth. If they are to open this wealth to exploration, it is only fair that they share equitably in the profits derived from the marketing of the wealth. Such has clearly not been the case in the past. Nor has there been

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an explicit policy of reinvesting a share of the profits derived from useful natural products in the preservation of nature's treasury.[1]

One would hope that as our Society moves into its seventh year, and gains in strength and influence, it would see fit to build upon this resolution. Chemical exploration in developing nations could be promoted by establishing or strengthening in these nations laboratories appropriately suited for such efforts. The initial step in chemical exploration is the discovery of biological activities associated with specific organisms or parts thereof. It would obviously be desirable and feasible to set up laboratories devoted to such chemical screening at or near sites slated for conservation. A network of such laboratories the world over, operating, it is hoped in some sort of coordination, could help enormously in promoting the chemical prospecting venture, while indirectly contributing to the conservation of nature. To move toward such a goal, a vigorous dialog will need to be established involving representatives of pharmaceutical and chemical industries, academic institutions, governments, and foundations. Our Society would do well to catalyze such interactions.

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[1] For a more detailed treatment of this topic see T. Eisner: "Prospecting for Nature's Chemical Riches," *Issues in Science and Technology 6*, 31–34 (1990).

Journal of Chemical Ecology, Vol. 16, No. 3, 1990

VOLATILE SEED GERMINATION INHIBITORS FROM PLANT RESIDUES[1]

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Abstract—Volatile emissions from residues of the winter cover legumes, Berseem clover (*Trifolium alexandrinum* L.), hairy vetch [*Vicia hirsuta* (L.) S.F. Gray], and crimson clover (*Trifolium incarnatum* L.), inhibited germination and seedling development of onion, carrot, and tomato. Using GC-MS, 31 C_2–C_{10} hydrocarbons, alcohols, aldehydes, ketones, esters, furans, and monoterpenes were identified in these residue emission mixtures. Mixtures of similar compounds were found in the volatiles released by herbicide-treated aerial and root residues of purple nutsedge (*Cyperus rotundus* L.) and the late-season woody stems and roots of cotton (*Gossypium hirsutum* L.). Vapor-phase onion, carrot, and tomato seed germination bioassays were used to determine the time- and concentration-dependent inhibition potential of 33 compounds that were either identified in the plant residue emissions or were structurally similar to identified compounds. Cumulative results of the bioassays showed that (*E*)-2-hexenal was the most inhibitory volatile tested, followed by nonanal, 3-methylbutanal, and ethyl 2-methylbutyrate. All the volatile mixtures examined contained at least one compound that greatly inhibited seed germination.

Key Words—Allelopathy, allelochemicals, *Allium cepa, Daucus carota, Lycopersicon esculentum*, volatiles, cover crops, conservation tillage, cotton, hydrocarbons, alcohols, ketones, aldehydes, furans, esters.

[1] Mention of a commercial or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture.

INTRODUCTION

Allelochemicals released into the soil from decomposing plant residues strongly impact the soil ecosystem, affecting the chemical environment of the entire detritus food web and rhizosphere (Elliott et al., 1978; Putnam, 1985; Hendrix et al., 1986). However, information is extremely limited concerning the chemical composition of plant residue emissions, particularly volatile compounds that affect the ecology of both higher plants (Putnam, 1985; Bradow and Connick, 1987, 1988c; Connick et al., 1987) and fungi (Lindermann and Gilbert, 1975; French, 1985; Gramss, 1985). The bioactive emissions from plant residues are a particularly important factor in conservation tillage practices that leave high levels of decomposing plant residues in the root zone and at the soil surface (Elliott et al., 1978; Phillips et al., 1980; Gebhardt et al., 1985; Putnam, 1985; Hendrix et al., 1986).

Few plant volatile emission profiles that represent plant residues are found in the literature. In insect semiochemical research a variety of host-plant volatile profiles have been determined (Metcalf, 1987) using harsh distillation procedures that produced volatile mixtures unrepresentative of natural plant residue emissions (Flath et al., 1978; Buttery and Kamm, 1980; Flath et al., 1984). By using prompt isolation from fresh plant material and gentler trapping techniques, other researchers obtained profiles more representative of growing plants and, depending on harvest technique, plant residues as well (Buttery et al., 1984, 1985, 1986; Buttery and Ling, 1984, 1985). Many of the compounds identified in these profiles have both insect semiochemical activity (Visser, 1979, 1983; Light et al., 1988), and also affect fungal growth and seed germination (French et al., 1977; French and Leather, 1979).

Germinating seeds are particularly vulnerable to chemicals released by plant residues into the soil atmosphere (Klarich and Weaver, 1973; Lill et al., 1979; Heisey and Delwiche, 1984; Bradow and Connick, 1987; 1988c; Oleszek, 1987). Cover crop residues and other plant debris have been reported to cause significant reductions in seedling survival and growth (Buntley, 1986; Dabney et al., 1986; Menges, 1987), as well as seed cotton yield reductions (Rickerl et al., 1988). Residues of the weed, Palmer amaranth (*Amaranthus palmeri* S. Wats.), release volatiles into the rhizosphere in sufficient quantity to inhibit the germination and seedling development of onion, carrot, and tomato and Palmer amaranth itself (Bradow and Connick, 1987). The volatile emissions profiles of one- and 15-day post harvest dried residues of *A. palmeri* (Connick et al., 1987) and *A. hybridus, A. cruentus, A. spinosus*, and *A. hypocondriacus* (Connick et al., 1989) have been reported, and the relative reductions in seed germination produced by the methyl ketones and related alcohols and aldehydes identified in those profiles have been described (Bradow and Connick, 1988a, b).

To provide further information necessary for the proper management of

crop residues in cover crop and reduced tillage situations, profiles of the volatiles emitted by residues of three winter cover crop legumes, Berseem clover (*Trifolium alexandrinum* L.), hairy vetch [*Vicia hirsuta* (L.) S.F. Gray], and crimson clover (*Trifolium incarnatum* L.), were determined. This paper also presents plant residue emission profiles from herbicide-treated residues of the common cotton-field weed, purple nutsedge (*Cyperus rotundus* L.) and end-of-season, mature woody cotton (*Gossypium hirsutum* L.) stems and roots. Vapor-phase seed germination bioassays of the effects of ethyl butyrate, geraniol, farnesol, and 31 compounds identified in these plant residue emission profiles were conducted.

METHODS AND MATERIALS

Plant Residues. The three winter cover crop legumes, *Trifolium alexandrinum* L. (Berseem clover cv. Bigbee), *Vicia hirsuta* (L.) S.F. Gray (hairy vetch), and *Trifolium incarnatum* L. (crimson clover cv. Tibbee), were inoculated with appropriate *Rhizobium* strains and planted after fall cotton plow-down in mid-December, 1986. The aerial portions were harvested in late March 1987, after flower anthesis and immediately before field preparation for the 1987 cotton crop.

Aerial and root portions of *Cyperus rotundus* L. (purple nutsedge) were harvested from between rows of *Gossypium hirsutum* L. (cotton cv. Stoneville 825) seven days after the application of the herbicide, MSMA (monosodium methane arsonate). Rainfall during the period between herbicide application and harvest was 1.3 cm.

Mature woody main stems and roots of *Gossypium hirsutum* L. (cotton, cv. Stoneville 825) were collected in mid-October 1987. The cotton had been hand-harvested and was not treated with defoliant.

Bioassay Materials. Chemicals used in the bioassays were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin) with the exception of a 4-methyl-2-pentanone and 5-methyl-2-heptanone, which were obtained from Polyscience Corp. (Nile, Illinois). Cover crop legume and assay seeds were purchased from commercial sources.

Volatiles Trapping Procedure. All nonwoody plant materials were cut into 0.5- to 2.5-cm pieces and allowed to air-dry in the laboratory for one or 15 days before analysis. Woody purple nutsedge roots and cotton stems and roots were cut into 1- to 3-cm pieces and pounded with a mallet before air-drying. Volatiles from the separate plant residues were collected as previously described (Connick, et al., 1987). A sample of plant residue (80 g if dried for one day or 20 g if dried for 15 days) was placed in a 1.2-liter glass solvent storage bottle with Teflon inlet and outlet valves. Ultrapurified water (10 ml) was added, the

valves closed, the bottle wrapped in aluminum foil, and the sample incubated for daily cycles of 10 hr at 31°C and 14 hr at 21°C. A glass tube (84 mm long × 9 mm OD × 1 mm wall thickness) packed with 0.1 g Tenax GC (60–80 mesh) and preconditioned at 220°C (24 hr, 15 ml/min He) was attached to the exit valve of the sample bottle and swept through with headspace atmosphere by purified nitrogen (20 min, 100 ml/min). Two Tenax tubes were collected sequentially for each plant residue. Volatile control profiles were collected using only 10 ml water in the sample bottle.

GC-MS Analysis. For separation and identification, plant residue volatiles were thermally desorbed from the Tenax tubes in an external, closed inlet system (Scientific Instrument Service, River Ridge, Louisiana) interfaced with a Perkin-Elmer Sigma 300 GC/Finnigan MAT Ion Trap Detector (ITD) model 700 unit (Connick et al., 1989). The capillary GC column was 50 m × 0.31 mm ID coated with 0.52 μm of 5% phenyl methyl silicone (Hewlett-Packard Ultra-2). Volatiles were desorbed according to the thermal protocol of Connick et al. (1989). Compounds were considered to be positively identified if their retention times and mass spectra matched those obtained using authentic compounds run on the GC-ITD under the same conditions as the residue volatile mixtures.

Time-Dependent Seed Germination Bioassays. The desiccator seed germination assay technique used is the same described previously (Bradow and Connick, 1987, 1988a, b; Connick et al., 1987). Seeds of onion (*Allium cepa* L., cv. Texas Early Grano 502), carrot (*Daucus carota* L., cv. Danvers Half-Long) or tomato (*Lycopersicon esculentum* Mill., cv. Homestead) were spread on double sheets of deionized water-saturated Whatman No. 1 filter paper placed on the porcelain plate of a 2.5-liter glass desiccator. Circles (22 mm diam.) had been removed from the filter paper to facilitate diffusion of volatiles, and the filter paper circles were divided into eight equal segments (replicates) containing 20 seeds each. Each desiccator well contained a 10-ml glass beaker (volatile source) resting on 50 g of pure sand in a crystallizing dish (100 × 50 mm). The sand was moistened with 10 ml of deionized water. The source beaker was left empty in the controls.

In the time-dependent studies, a volume equivalent to 34.4 μM/liter (full volatilization assumed) of the volatile test compound was placed undiluted in the source beaker, and the separate seed species were incubated under the same temperature regimen used in the volatile-trapping procedure for 72 hr before germination evaluation (three-day data). Radicle protrusion was the germination criterion. The source beaker was then either removed (three- + four-day data) or the test volatile was renewed by the addition a second 34.4 μM equivalent (seven-day data). All seeds were then incubated an additional four days before final evaluation. Each assay was performed two times (16 replicates, total), and controls (deionized water) were repeated at three-month intervals throughout

the duration of the experiments. Germination of the controls did not change significantly during the course of the experiment (data not shown).

Concentration-Dependent Seed Germination Bioassays. The source beaker of the desiccator apparatus contained the appropriate volume of a single test compound to give a nominal volatile concentration of 6.9, 20.8, or 34.4 μM/liter, assuming total volatilization and negligible adsorption. The volume of each test volatile was adjusted on the basis of the compound density and added undiluted to the source beaker, using a microliter syringe (Bradow and Connick, 1988a). Seed germination was evaluated after a three-day exposure to a test volatile, the volatile source was removed, and the seeds were incubated for an additional four days. In both types of bioassays, the humidity within the desiccators was maintained by the addition of 10 ml deionized water after the three-day germination evaluation.

Statistical Analyses. After normalization by the transformation $(x + 0.5)^{0.5}$, germination count data from the concentration-dependent bioassays and the seven-day and three- + four-day time-dependent assays were compared separately for each seed species by two-way analyses of variance with 16 replicates. Within the three-day, seven-day, and three- + four-day data, one-way analyses of variance were used to determine significant differences between the mean effects of 34.4 μM/liter of the test compounds and appropriately incubated deionized water controls, using Tukey's Honestly Significant Difference procedure (significance level, $P = 0.01$). The concentration dependence data were examined by regression analysis (Sokal and Rohlf, 1981). To permit comparisons with data from previous papers (Bradow and Connick, 1988a, b), one-way analyses of variance were performed on the data from the time dependence (34.4 μM) bioassays of all volatiles tested for this and the other publications.

RESULTS

Volatile Emissions from Plant Residues. The compounds identified in the emissions from the residues of the three winter legumes, purple nutsedge, and cotton roots and stems are shown in Table 1. Two branched-chain alcohols, 2-methyl-1-propanol and 3-methyl-1-butanol, were the only compounds found in all the volatile mixtures examined. Acetaldehyde appeared to be present in the emissions from all nonwoody residues, but the GC retention time was too short to allow positive identification (Connick et al., 1989). Small, but identifiable, amounts of *n*-pentane, ethanol, methylene chloride, chloroform, and benzene were found in the water controls, indicating that the strong *n*-pentane peaks obtained from residues of the legumes and nutsedge may be a partial artifact. The *Z* and *E* forms of 3-hexen-1-ol and 4-methyl-2-pentene isomers were not separated, but both isomers were bioassayed.

TABLE 1. VOLATILE COMPOUNDS EMITTED BY RESIDUES OF WINTER COVER CROP LEGUMES, NUTSEDGE, AND WOODY COTTON ROOTS AND STEMS

	Source residue[a]					
				Nutsedge		
	Berseem aerial	Vetch aerial	Crimson aerial	Aerial	roots	Cotton stems/roots
Hydrocarbons						
n-Pentane	++	++	++	++	+	–
n-Hexane	+	+	–	–	–	–
n-Octane	–	+	++	+	–	–
n-Nonane	++	+	+	++	–	–
4-Me-2-Pentene[b]	–	+	–	++	–	–
Alcohols						
2-Propanol	+	–	++	–	–	+
1-Propanol	–	+	++	+	+	+
2-Me-1-propanol	++	++	++	++	+	++
2-Butanol	++	+	++	+	–	–
1-Pentanol	–	–	–	++	–	–
2-Pentanol	++	–	+	++	–	–
3-Me-1-butanol	++	++	++	+	+	++
1-Hexanol	++	+	+	+	–	–
3-Hexen-1-ol[b]	+	+	+	+	–	–
Aldehydes						
Acetaldehyde	+	+	+	+	–	–
Pentanal	–	++	+	–	–	–
2-Me-Butanal	–	+	+	+	–	–
3-Me-Butanal	–	+	+	+	–	–
(*E*)-2-Hexenal	–	–	–	+	–	–
Nonanal	–	–	–	+	–	–

Ketones						
2-Butanone	++	++	++	++	+	+
2-Pentanone	−	−	−	+	+	+
3-Pentanone	++	+	++	+	−	−
3-Me-2-butanone	−	+	++	++	−	−
4-Me-2-pentanone	−	−	−	+	+	−
2-Heptanone	+	+	+	+	+	−
-5-Me-2-heptanone	−	−	+	+	−	−
3-Octanone	+	+	+	−	−	−
2-Nonanone	++	++	+	−	−	−
Esters						
Methyl acetate	++	++	++	−	−	−
Ethyl acetate	++	+	−	+	+	++
Ethyl propionate	++	+	++	+	−	−
Ethyl isobutyrate	−	−	−	+	+	+
Ethyl 2-Me-butyrate	−	−	−	−	+	+
Furans						
2-Methylfuran	−	++	++	++	++	−
2-Ethylfuran	++	+	++	++	+	−
Terpenes						
D-Limonene	−	+	+	++	++	++
α-Pinene	−	−	−	+	++	+
β-Myrcene	−	−	−	+	+	+

[a] Observed: [+]; major peak: [++]; not observed: [−].
[b] Specific isomer not determined.

The following compounds were found in the volatile profiles from the three cover crop legumes: *n*-pentane, *n*-nonane, 2-methyl-1-propanol, 2-butanol, 3-methyl-1-butanol, 1-hexanol, 3-hexen-1-ol, 2-butanone, 3-pentanone, 2-heptanone, 3-octanone, 2-nonanone, methyl acetate, ethyl propionate, and 2-ethylfuran. The volatile mixtures from the two clovers, but not the vetch, also contained 2-propanol and 2-pentanol. The volatiles emitted by vetch contained 4-methyl-2-pentene, which was not found in the emissions from the legumes but did occur in those from aerial nutsedge.

Aerial purple nutsedge volatile emissions were similar to those of the three winter legumes. Of the compounds listed above as ubiquitous in the legume emissions, only 3-octanone, 2-nonanone, and methyl acetate were not present in the aerial nutsedge mixtures. 4-Methyl-2-pentene, 1-pentanol, nonanal, and *trans*-2-hexenal were unique to aerial nutsedge; and 4-methyl-2-pentene, 2-pentanone, α-pinene, and β-myrcene were emitted by aerial and root parts of nutsedge, but not by the winter legumes. Nutsedge aerial and root portions released more terpenes than did the winter legumes. Drying the plant residues for 15 days altered the volatile profiles quantitatively, but not qualitatively.

The woodier tissues, the purple nutsedge roots, and the cotton roots and stems qualitatively and quantitatively emitted fewer volatiles than did the more succulent green aerial plant parts. Extensive maceration, in contrast to simple chopping, was necessary to release identifiable levels of volatiles from the woodier tissues at the ambient temperatures used in these experiments. Ethyl 2-methylbutyrate was unique to the woody nutsedge roots and cotton. Pentane, 2-heptanone, 2-butanone, and 2-ethylfuran appeared in the every volatile profile examined except that of cotton wood.

Effects of Plant Residue Volatiles on Seed Germination. The generation effects of five methyl ketones (Bradow and Connick, 1988a), three alcohols, acetaldehyde, and 3-pentanone (Bradow and Connick, 1988b), listed in Table 1, have been described previously. The terpenoids, geraniol and farnesol, occur as unstable pyrophosphates in the mevalonate pathway, which is ubiquitous in plants (Goodwin and Mercer, 1983), and were included in the bioassays as examples of monoterpenoid and sesquiterpenoid alcohols, but neither compound was detected in any volatile profile examined. The *Z* and *E* isomers of 3-hexen-1-ol and 4-methyl-2-pentene were assayed separately to permit examination of the effects of hydrocarbon chain isomerization. Ethyl butyrate was not detected in any of the volatile emission mixtures tested, but it was included to allow structure–activity comparisons with ethyl isobutyrate and ethyl 2-methylbutyrate, which were detected in the volatiles from purple nutsedge and cotton.

Onion Seed Germination. After a three-day exposure to 6.9 μM, the vapors of all test compounds except 3-methylbutanal, 4-methyl-2-pentanone, 5-methyl-2-heptanone, and 3-octanone significantly inhibited onion seed germination, compared to the water controls (Table 2). The most inhibitory compound at this

concentration was (*E*)-2-hexenal. Germination was reduced to 50% of that observed in the control by the hydrocarbons *n-pentane, n*-octane, and *n*-nonane; both 4-methyl-2-pentene isomers; the alcohols 1-propanol, 2-propanol, 1-pentanol, and (*Z*)-3-hexen-1-ol; the aldehydes pentanal, 2-methybutanal, (*E*)-2-hexenal, and nonanal; the esters methyl acetate, ethyl propionate, ethyl isobutyrate, ethyl butyrate, and ethyl 2-methybutyrate; by both furans, and by all five terpenoids.

When concentrations of test volatiles were increased to 20.8 μM, only 4-methyl-2-pentanone and 5-methyl-2-heptanone did not significantly inhibit onion germination. Exposure for three days to 34.4 μM of the test volatiles, *n*-octane, *n*-nonane, both 4-methyl-2-pentene isomers, 3-octanone, and all alcohols, aldehydes, esters, furans, and terpenoids from Table 2, reduced onion seed germination to less than 50% of control germination. At the lowest concentration (*E*)-2-hexenal prevented onion germination entirely; and (*Z*)-3-hexen-1-ol, 3-methylbutanal, nonanal, and pentanal were nearly as inhibitory, followed by ethyl butyrate and ethyl isobutyrate.

Regression analysis showed that, when the data for the water control were included, the concentration dependence of the effects of each of the test compounds had a significant linear component. However, flat responses to most of the compounds over the assay concentration range used contributed significant regression deviation from the linear. Simple linear concentration-dependent effects were seen only in onion seeds exposed for three days to n-hexane, (*E*)-3-hexen-1-ol, and ethyl acetate. Increasing concentrations of 4-methyl-2-pentanone and 5-methyl-2-heptanone had no effect on onion germination. There were no significant changes in slope in the three- + four-day concentration-dependent data, which have, therefore, been omitted from this table.

When the volatile source was removed after the three-day exposure and seed germination evaluated under an exhausting safety hood, an additional four-day incubation allowed onion seeds exposed to all test compounds, except 1-propanol, (*Z*)-hexen-1-ol, pentanal, 3-methylbutanal, (*E*)-2-hexenal, nonanal, ethyl acetate, ethyl propionate, ethyl butyrate, and ethyl 2-methylbutyrate, to germinate as fully as the water controls. After this additional incubation in the absence of the test volatile, germination of only those seeds originally exposed to 3-methylbutanal, hexenal, and ethyl butyrate was below 50% of the germination observed in the water controls after a seven-day incubation period. These same three compounds, plus ethyl 2-methylbutyrate, reduced onion seed germination to less than 50% of water control germination after a continuous seven-day exposure. Continuous seven-day exposure to all the hydrocarbons, 2-propanol, 2-butanol, (*Z*)- and (*E*)-3-hexen-1-ol, pentanal, nonanal, 2-ethylfuran, and β-myrcene also significantly inhibited onion germination.

Carrot Seed Germination. Carrot seeds were less sensitive than onion seeds to a three-day exposure to 6.9 μM levels of the volatiles (Table 3). Only *n*-pentane, (*E*)-2-hexenal, nonanal, the three ketones, ethyl butyrate, ethyl 2-

TABLE 2. EFFECTS OF VOLATILE EXPOSURE DURATION AND CONCENTRATION ON GERMINATION OF ONION SEEDS

	Exposure time				
	3 Days			3 + 4 Days	7 Days
	Germination (%)[a] at				
	6.9 μM	20.8 μM	34.4 μM	34.4 μM	34.4 μM
Hydrocarbons					
n-Pentane	22.0 c–j	23.7 h–k	28.0 h	54.2 e–l	56.7 f–j
n-Hexane	39.1 kl	34.9 lm	28.9 h	58.3 e–m	57.1 f–j
n-Octane	26.2 ij	27.3 j–l	26.3 gh	57.2 e–m	57.3 f–k
n-Nonane	20.1 b–i	20.1 e–j	18.1 d–h	52.9 d–k	40.1 cd
(*Z*)-4-Me-2-Pentene	17.5 b–g	19.4 e–j	26.6 gh	74.5 mn	53.2 fg
(*E*)-4-Me-2-Pentene	17.7 b–h	19.7 e–j	21.3 e–h	78.3 n	55.5 f–h
Alcohols					
1-Propanol	24.1 e–j	23.3 h–k	18.5 d–h	44.2 c–e	65.4 j–l
2-Propanol	21.0 b–i	15.8 c–i	14.8 c–f	63.9 h–n	56.4 f–i
2-Butanol	37.0 kl	22.7 g–j	21.7 f–h	69.4 k–n	56.4 f–j
1-Pentanol	23.5 d–j	20.0 e–j	10.2 c–e	60.3 f–m	66.4 l
(*Z*)-3-Hexen-1-ol	19.0 b–i	5.3 b	0.2 a	47.7 c–g	49.2 ef
(*E*)-3-Hexen-1-ol	38.0 kl	27.4 j–l	16.6 d–h	52.9 d–k	44.3 de
Aldehydes					
Pentanal	16.4 b–e	13.1 c–f	1.9 ab	46.6 c–f	37.2 cd
2-Methylbutanal	18.5 b–i	16.2 c–i	15.0 c–g	70.2 l–n	66.2 kl
3-Methylbutanal	44.8 lm	34.7 lm	0.9 ab	15.0 b	0.0 a
(*E*)-2-Hexenal	4.8 a	0.5 a	0.0 a	0.2 a	0.0 a
Nonanal	19.5 b–i	11.3 b–e	1.7 ab	62.8 g–h	42.0 c–e

Ketones					
4-Me-2-pentanone	53.7 m	51.5 n	53.1 i	67.8 i–n	67.5 l
5-Me-2-heptanone	53.1 m	46.6 mn	51.8 i	66.8 i–n	65.2 i–l
3-Octanone	52.2 m	33.3 kl	22.8 f–h	64.8 i–n	64.9 i–l
Esters					
Methyl acetate	24.2 e–j	23.1 h–k	21.0 e–h	65.3 i–n	63.3 h–l
Ethyl acetate	30.7 jk	23.3 h–k	21.2 e–h	50.6 c–i	66.4 l
Ethyl propionate	22.4 c–j	17.6 d–i	16.6 d–h	38.2 c	64.9 i–l
Ethyl isobutyrate	14.1 b	10.9 b–d	9.3 cd	63.8 h–n	63.5 h–l
Ethyl butyrate	16.8 b–f	8.9 bc	6.8 bc	39.0 cd	36.8 c
Ethyl 2-Me-butyrate	15.9 b–d	12.6 c–f	10.2 c–e	48.3 c–h	18.2 b
Furans					
2-Methylfuran	15.5 bc	14.1 c–g	14.6 c–g	68.7 k–n	65.3 j–l
2-Ethylfuran	24.8 f–j	22.8 g–k	18.4 d–h	51.7 c–j	53.7 fg
Terpenoids					
D-Limonene	26.0 h–j	24.5 i–l	22.9 f–h	62.2 f–n	60.9 g–l
α-Pinene	25.1 g–j	22.5 g–j	21.3 e–h	67.9 j–n	62.7 h–l
β-Myrcene	21.5 b–i	20.6 e–j	19.5 d–h	68.8 k–n	56.9 f–j
Geraniol	22.8 c–j	15.0 c–h	13.0 c–f	59.8 f–m	63.8 h–l
Farnesol	24.4 e–j	22.6 g–j	17.8 d–h	60.5 f–n	68.8 l
Control	53.4 m	53.4 n	53.4 i	67.8 j–n	67.8 l

[a] Percentages are the means of 16 replications. Values in columns followed by the same letter and associated with a given exposure time are not significantly different ($P = 0.01$). Standard errors of the means were $<2.8\%$.

TABLE 3. EFFECTS OF VOLATILE EXPOSURE DURATION AND CONCENTRATION ON GERMINATION OF CARROT SEEDS

	Exposure time				
	3 Days			3 + 4 Days	7 Days
	Germination (%)[a] at				
	6.9 μM	20.8 μM	34.4 μM	34.4 μM	34.4 μM
Hydrocarbons					
n-Pentane	18.7 b–e	21.6 g–i	27.0 kl	73.8 cd	71.9 e–h
n-Hexane	39.5 lm	40.0 m	19.9 h–k	81.8 cd	76.4 g–i
n-Octane	38.5 k–m	38.3 lm	26.4 j–l	75.7 cd	79.5 h–j
n-Nonane	32.3 g–m	29.5 i–l	27.3 kl	79.5 cd	83.5 i–k
(*Z*)-4-Me-2-Pentene	25.7 b–k	32.7 k–m	47.1 n	77.8 cd	87.7 jk
(*E*)-4-Me-2-Pentene	28.0 d–l	28.5 i–k	21.9 i–l	79.1 cd	91.1 k
Alcohols					
1-Propanol	36.1 j–m	26.3 i–k	18.5 g–k	77.4 cd	73.3 f–h
2-Propanol	28.4 d–l	17.5 f–h	16.4 f–j	79.5 cd	79.6 h–j
2-Butanol	46.7 m	32.9 k–m	22.5 i–l	81.7 cd	80.0 h–j
1-Pentanol	26.3 c–l	23.4 g–j	10.0 d–f	78.9 cd	74.1 g–i
(*Z*)-3-Hexen-1-ol	22.1 b–h	16.5 f–h	0.0 a	78.3 cd	74.8 g–j
(*E*)-3-Hexen-1-ol	30.6 f–l	27.3 i–k	2.5 a–c	75.2 cd	72.3 g–i
Aldehydes					
Pentanal	31.4 f–l	26.9 i–k	6.0 b–d	75.1 cd	72.9 e–h
2-Methylbutanal	34.8 i–m	24.2 h–j	12.6 d–h	83.8 d	69.7 d–g
3-Methylbutanal	29.8 e–l	23.5 h–j	0.0 a	23.7 b	5.2 b
(*E*)-2-Hexenal	3.6 a	0.0 a	0.0 a	0.0 a	0.0 a
Nonanal	16.0 bc	4.4 bc	1.2 ab	28.8 b	1.5 a

Ketones					
4-Me-2-pentanone	15.9 bc	9.5 d	7.4 c–g	78.5 cd	70.0 d–g
5-Me-2-heptanone	15.0 b	11.4 d–f	7.2 c–f	76.7 cd	75.9 g–i
3-Octanone	17.0 b–d	16.0 e–g	14.1 e–i	73.0 cd	61.4 d
Esters					
Methyl acetate	30.2 e–l	23.3 g–j	22.5 i–l	80.7 cd	81.7 h–k
Ethyl acetate	34.1 h–m	28.7 i–k	22.2 i–l	78.2 cd	77.7 g–i
Ethyl propionate	33.9 h–m	7.3 cd	0.5 a	63.8 c	21.0 c
Ethyl isobutyrate	21.8 b–h	10.4 de	3.5 a–c	81.0 cd	65.0 d–f
Ethyl butyrate	17.9 b–d	1.3 ab	2.3 a–c	72.1 cd	64.0 de
Ethyl 2-Me-butyrate	19.8 b–f	16.3 e–g	15.7 f–i	79.8 cd	61.6 d
Furans					
2-Methylfuran	21.3 b–g	12.2 d–f	10.9 d–g	88.6 d	73.6 f–h
2-Ethylfuran	15.0 b	21.9 g–i	28.6 k–m	74.2 cd	79.6 h–j
Terpenoids					
D-Limonene	29.7 e–l	28.6 i–k	12.8 d–h	76.6 cd	76.6 g–i
α-Pinene	24.1 b–j	27.9 i–k	39.8 mn	75.8 cd	76.5 g–i
β-Myrcene	23.2 b–i	23.9 h–j	26.0 j–l	78.2 cd	81.4 h–k
Geraniol	22.8 b–i	17.6 f–h	15.7 f–i	84.1 d	77.3 g–i
Farnesol	25.7 b–k	26.3 i–k	22.9 i–e	74.9 cd	74.4 g–i
Control	30.6 f–l	30.6 j–l	30.6 lm	77.0 cd	77.0 g–i

[a] Percentages are the means of 16 replications. Values in columns followed by the same letter and associated with a given exposure time are not significantly different ($P = 0.01$). Standard errors of the means were $< 1.8\%$.

methylbutyrate, and 2-ethylfuran significantly inhibited germination at that concentration. When carrot seeds were exposed to 20.8 μM of the test volatiles, germination was reduced to 50% of the control values by (*E*)-2-hexenal, nonanal, 4-methyl-2-pentanone, 5-methyl-2-heptanone, ethyl propionate, ethyl isobutyrate, ethyl butyrate, and 2-methylfuran. At this concentration *n*-pentane, 2-propanol, (*Z*)-3-hexen-1-ol, 3-octanone, ethyl 2-methylbutyrate, 2-ethylfuran, and geraniol also significantly inhibited carrot seed germination. When the concentrations were increased to 34.4 μM, all volatiles tested except the four saturated alkanes, the two 4-methyl-2-pentene isomers, 2-butanol, methyl acetate, ethyl acetate, 2-ethylfuran, α-pinene, β-myrcene, and farnesol significantly inhibited carrot seed germination. A three-day exposure to 1-pentanol, both 3-hexen-1-ol isomers, pentanal, (*E*)-2-hexenal, 2-methylbutanal, 3-methylbutanal, nonanal, all three ketones, ethyl propionate, ethyl isobutyrate, ethyl butyrate, 2-methylfuran, and limonene reduced carrot seed germination percentages to below 50% of the control values.

Regression analyses showed that the response of carrot seed to increasing volatile concentrations were linear in the case of 2-propanol, 1-pentanol, the three ketones, methyl acetate, ethyl acetate, ethyl isobutyrate, ethyl 2-methylbutyrate, 2-methylfuran, and geraniol. Increasing concentrations of *n*-nonane, (*E*)-4-methyl-2-pentene, and farnesol had no effect, and increasing the concentration of *n-pentane,* (*Z*)-4-methyl-2-pentene, 2-ethylfuran, and α-pinene from 6.9 to 34.4 μM reduced the inhibitory effect of exposure to these compounds and produced nonlinear response curves. The concentration-dependence regressions obtained for all other test compounds in the carrot seed bioassay had both significant linear and nonlinear components.

When the volatiles source was removed and the carrot seeds were incubated an additional four days, the initial three-day exposure to 34.4 μM (*E*)-2-hexenal proved sufficient to completely prevent carrot seed germination. Of the other test compounds, only 3-methylbutanal and nonanal residually inhibited carrot seed germination. There were no residual effects from exposure to lower concentrations of any of the compounds except (*E*)-2-hexenal, 3-methylbutanal, and nonanal, and the slopes of carrot seed responses to those three compounds did not change over the additional four days of incubation (data not shown). Continuous exposure for seven days to the two 4-methyl-2-pentene isomers increased carrot seed germination to levels significantly higher than the germination percentage of the control. The three compounds that had a residual effect on carrot seed germination, plus ethyl propionate, reduced carrot seed germination to less than 50% of the control value after a seven-day continuous exposure. 3-Octanone, ethyl isobutyrate, ethyl butyrate, and ethyl 2-methylbutyrate also significantly inhibited carrot seed after a seven-day exposure.

Tomato Seed Germination. A three-day exposure to 6.9 μM (*E*)-3-hexen-1-ol, 4-methyl-2-pentanone, 5-methyl-2-heptanone, methyl acetate, 2-ethyl-

furan, α-pinene, and geraniol reduced tomato seed germination to less than 50% of the germination observed in the water controls (Table 4). Significant inhibition was also observed in tomato seeds exposed to the same concentration of *n*-pentane, *n*-octane, 2-methylbutanal, nonanal, ethyl propionate, 2-methylfuran, limonene, and β-myrcene. At 20.8 μM, a three-day exposure to all of these compounds plus 2-butanol, (*Z*)-3-hexen-1-ol, pentanal, (*E*)-2-hexenal, 3-methylbutanal, (*E*)-2-hexenal, 3-octanone, ethyl isobutyrate, ethyl butyrate, and ethyl 2-methylbutyrate significantly inhibited the germination of tomato seeds. At 34.4 μM, all the test compounds except *n*-nonane, the two methyl pentene isomers, ethyl acetate, ethyl propionate, 2-ethylfuran, and farnesol inhibited tomato germination significantly. Significant residual inhibition after the removal of the volatile source and an additional four-day incubation was observed in tomato seeds initially exposed for three days to (*E*)-2-hexenal and nonanal. There were no significant residual effects observed in tomato seeds exposed to 6.9, 20.8, or 34.4 μM levels of any of the other compounds, and the slopes of the tomato seed residual responses to the three levels of (*E*)-2-hexenal and nonanal did not differ from those obtained after the initial three-day exposure (data not shown). Continuous seven-day exposure to 3-methyl butanal, (*E*)-2-hexenal, nonanal, 3-octanone, ethyl butyrate, and ethyl 2-methylbutyrate significantly inhibited tomato germination, but only the hexenal and nonanal reduced germination to below 50% of the control.

The only linear responses to increasing volatile concentration were observed when tomato seeds were exposed to *n*-hexane, *n*-octane, 2-methylfuran, and limosene for three days. Null concentration effects were observed in tomato seeds exposed to *n*-nonane, and the two 4-methyl-2-pentene isomers. All other tomato seed concentration response regressions had significant linear components and significant deviations from linearity.

DISCUSSION

The literature offers few direct comparisons with volatile emission profiles obtained for plant residues through passive Tenax trapping techniques. The "green plant" volatiles identified in the leaf odor blends from Solanaceae (Visser et al., 1979; Visser, 1983) and Cruciferae (Wallbank and Wheatley, 1976) contained significant levels of straight chain C_6 saturated and unsaturated alcohols, aldehydes, and the derivative acetates. In plant residue emissions, hexanal was identified in the volatile profile of *Amaranthus palmeri* (Connick et al., 1987). By comparison, 1-hexanol and the 3-hexen-1-ol isomers were found in the profiles obtained from the aerial parts of the three legumes and purple nutsedge, and (*E*)-2-hexenal was identified in the volatiles emitted by the nutsedge aerial parts (Table 1). The only compounds found in the volatiles released by

TABLE 4. EFFECTS OF VOLATILE EXPOSURE DURATION AND CONCENTRATION ON GERMINATION OF TOMATO SEEDS

	Exposure time				
	3 Days			3 + 4 Days	7 Days
	Germination (%)[a] at				
	6.9 μM	20.8 μM	34.4 μM	34.4 μM	34.4 μM
Hydrocarbons					
n-Pentane	40.9 f–i	35.8 i–m	16.6 d–g	95.3 c	95.6 f
n-Hexane	58.5 k–n	43.4 j–o	37.8 h–k	95.6 c	95.6 f
n-Octane	48.2 g–l	23.8 f–i	11.1 c–e	95.9 c	96.5 f
n-Nonane	60.3 l–n	58.8 n–p	65.2 lm	96.8 c	94.3 f
(*Z*)-4-Me-2-Pentane	61.7 l–n	67.0 op	71.9 m	96.5 c	94.9 f
(*E*)-4-Me-2-Pentene	61.4 l–n	59.9 n–p	53.6 j–m	95.6 c	94.6 f
Alcohols					
1-Propanol	66.7 mn	62.3 op	28.6 f–i	94.3 bc	94.7 f
2-Propanol	59.7 k–n	56.9 m–p	33.4 g–j	91.8 bc	95.2 f
2-Butanol	57.1 j–n	35.2 i–l	20.8 d–h	95.6 c	93.7 f
1-Pentanol	65.8 l–n	50.6 k–p	8.1 b–d	93.7 bc	91.8 ef
(*Z*)-3-Hexen-1-ol	61.4 l–n	9.3 b–e	0.0 a	88.6 bc	90.9 ef
(*E*)-3-Hexen-1-ol	26.8 d–f	23.8 f–i	1.5 ab	95.6 c	92.7 ef
Aldehydes					
Pentanal	67.9 mn	32.1 h–k	10.9 c–e	94.9 bc	93.1 ef
2-Methylbutanal	40.3 f–i	6.6 a–e	2.0 a–c	88.9 bc	94.0 f
3-Methylbutanal	52.9 h–m	5.6 a–e	2.2 a–c	92.1 bc	72.2 d
(*E*)-2-Hexenal	67.0 mn	0.0 a	0.0 a	25.1 a	13.1 b
Nonanal	43.4 f–k	3.7 a–c	0.0 a	82.7 b	0.5 a

Ketones					
4-Me-2-pentanone	32.8 d-f	2.2 ab	0.7 ab	95.6 c	95.5 f
5-Me-2-heptanone	6.0 a	1.1 ab	0.0 a	93.1 bc	85.1 ef
3-Octanone	54.0 i-m	8.6 b-e	0.0 a	94.3 bc	51.8 c
Esters					
Methyl acetate	8.7 ab	11.4 c-f	22.8 e-h	95.8 c	93.9 f
Ethyl acetate	56.0 i-n	67.9 p	78.2 m	93.1 bc	93.0 ef
Ethyl propionate	36.9 e-g	38.5 i-n	56.7 k-m	92.4 bc	93.7 f
Ethyl iosbutyrate	61.8 l-n	27.9 g-i	1.3 ab	96.8 c	95.3 f
Ethyl butyrate	59.9 l-n	27.9 g-i	16.9 d-g	92.3 bc	88.3 ef
Ethyl 2-Me-butyrate	73.5 n	4.2 a-d	0.0 a	94.6 bc	80.2 de
Furans					
2-Methylfuran	37.4 e-h	33.3 i-l	20.2 d-h	90.4 bc	95.5 f
2-Ethylfuran	24.8 c-e	43.5 j-o	44.1 i-l	95.9 c	90.2 ef
Terpenoids					
D-Limonene	42.2 f-j	36.4 i-m	28.4 f-i	95.6 c	96.8 f
α-Pinene	22.7 cd	16.5 e-h	17.3 d-g	96.8 c	88.3 ef
β-Myrcene	34.8 d-g	29.1 g-j	17.3 d-g	96.2 c	89.6 ef
Geraniol	15.5 i-m	14.2 d-g	14.7 d-f	95.0 bc	97.8 f
Farnesol	53.9 i-m	53.4 l-p	66.3 lm	97.5 c	96.8 f
Control	66.4 mn	66.4 no	66.4 lm	97.5 c	96.8

[a] Percentages are the means of 16 replications. Values in columns followed by the same letter and associated with a given exposure time are not significantly different ($P = 0.01$). Standard errors of the means were $< 1.5\%$.

freshly harvested aerial parts of red clover (*Trifolium pratense* L.) that also occur in the Berseem and crimson clover residue volatiles were 2-heptanone, 1-hexanol, and 3-hexen-1-ol (Buttery et al., 1984).

The C_6 compounds ubiquitous in leaf odors hypothetically arise from the oxidation of plant leaf lipids (Visser and Ave, 1978), probably through the action of endogenous lipoxygenase and alcohol dehydrogenase (Eriksson, 1975). In contrast, the plant residue emission mixtures contained mostly methyl ketones, alcohols with branched chains or chains of more than six carbons, and relatively few aldehydes (Connick et al., 1987, 1989). None of the compounds identified in the emissions of woody cotton residues were found in the "air space" of growing cotton plants (Hedin et al., 1975b). Plant residue profiles also changed when the pretrapping incubation was anaerobic, but the residue emissions did not change qualitatively with time after an initial air-drying for one day (Connick et al., 1987). The volatiles emitted by plant material clearly depend qualitatively on whether the plant is growing, freshly harvested, or decomposing aerobically or anaerobically.

The woodiness of the tissue and the method of collection also greatly influenced the plant volatile profiles obtained. Steam distillation of green cotton bracts produced a volatile mixture that contained, along with a large number of other compounds, α-pinene, β-myrcene, and 3-methyl-1-butanol (Hedin et al., 1975a). These three compounds were also detected in the volatiles passively emitted by cotton wood. Nonanal, 1-pentanol, (*E*)-2-hexenal, 1-hexanol, and (*Z*)-3-hexen-1-ol were detected in steam distillates from fresh cotton buds (Hedin et al., 1975a) but not in the passive emissions from cotton wood. Fewer compounds were detected using passive Tenax trapping than with harsher distillation techniques (Buttery et al., 1982), but Tenax-trapped volatiles are more representative of natural emissions from both green plants and plant residues. As observed with aerial and root nutsedge emissions (Table 1), the root tissue of corn emitted fewer compounds than does aerial tissue (Buttery and Ling, 1984, 1985).

Although the bioassay species differed in sensitivity to the different volatile compounds, the most inhibitory compound tested was (*E*)-2-hexenal, an emission from residues of aerial nutsedge. Hexenal has also been reported as a general "green plant" volatile (Visser et al., 1979) and identified in the steam distillate from *Amaranthus retroflexus* (Flath et al., 1984). Comparison of time- and concentration-dependent data from this and other publications (Bradow and Connick, 1988a, b) show (*E*)-2-hexenal to be significantly more inhibitory than 2-octanone, 2-nonanone, and 2-heptanone, the most inhibitory volatiles from plant residues reported previously.

In general, using the combined data from this and two previous papers (Bradow and Connick, 1988a, b), aldehydes were the most inhibitory of onion and carrot seed germination, and tomato seeds were most sensitive to ketones.

Onion seeds were more affected by the presence of volatile esters, particularly ethyl 2-methylbutyrate and ethyl butyrate, than were carrot and tomato seeds. Tomato seeds were not sensitive to terpenoid vapors than were onion and carrot seeds. The inhibitory activity of a specific volatile compound was related to the nature and position of the functional groups present and the length and degree of unsaturation of the hydrocarbon chain.

Decreases and retardations of seed germination are not the only ecologically significant effects that can be attributed to allelochemicals emitted into the soil atmosphere by plant residues. Some of the inhibitory compounds characterized in this and earlier papers (Bradow and Connick, 1988a, b) also stimulate the germination of competitive weeds (French and Leather, 1979; French et al., 1986) and the spores of pathogenic fungi (French, 1984; French et al., 1986). Volatiles from plant residues can also stimulate sclerotial (King and Coley-Smith, 1968; Linderman and Gilbert; 1969) and mycelial growth (Menzies and Gilbert, 1967). Many of these same compounds that retard seed germination and promote fungal growth are also insect attractants (Visser, 1979; 1983; Visser et al., 1979; Light et al., 1988) and retard seedling root elongation (Bradow and Connick, 1988c). Thus, seeds and seedlings already stressed by the presence of plant residue volatiles in the root zone become more susceptible to attack by fungi and insects.

Our results show that the "green plant" volatiles emitted by very fresh plant residues are extremely inhibitory to seed germination. This indicates that any tillage practice that incorporates large amounts of fresh green cover crop into the seeding zone immediately prior to planting should be avoided. Furthermore, dried plant residues, after rehydration, emitted detectable levels of inhibitory volatiles for more than four months after harvest (Bradow and Connick, 1987). The even-number carbon chain methyl ketones released from plant residues under anaerobic conditions are among the most inhibitory volatile allelochemicals. Volatile emissions from plant residues may have a decisive role in the success of any agricultural practice that involves incorporation of large quantities of plant material into the soil.

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STRUCTURE–ACTIVITY RELATIONSHIPS FOR CHAIN-SHORTENED ANALOGS OF (Z)-5-DECENYL ACETATE, A PHEROMONE COMPONENT OF THE TURNIP MOTH, *Agrotis segetum*[1]

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Abstract—Structure–activity relationships for chain-shortened analogs of (Z)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, have been studied by electrophysiological single-sensillum technique and interpreted in terms of a previously reported receptor–interaction model. The results indicate that the terminal methyl group, as well as the acetate group, interacts with highly complementary receptor sites. The terminal alkyl chain is suggested to interact with a hydrophobic "pocket" extending over the two methylene groups closest to the terminal methyl group. The amounts of stimulus actually released from the odor source have been studied. The results demonstrate the necessity to take differences of volatility into account in comparisons of electrophysiological data for compounds of different chain lengths. It is shown that relative vapor pressures may to a good approximation be employed to estimate correction factors.

Key Words—Structure–activity, single-cell recordings, receptor interaction, (Z)-5-decenyl acetate, chain-shortened analogs, volatility, vapor pressure, *Agrotis segetum*, Lepidoptera, Noctuidae.

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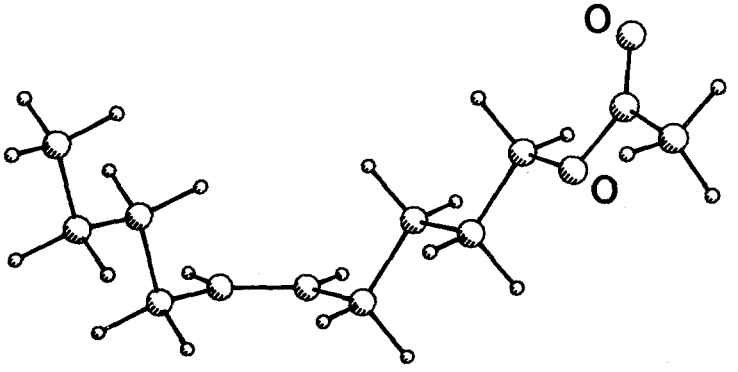
INTRODUCTION

In a series of papers, we have reported the development and applications of a model for the interaction between a monoolefinic pheromone component and its receptor, employing structures and conformational energies calculated by the molecular mechanics method (Liljefors et al., 1984, 1985, 1987; Bengtsson et al., 1987). This model has been successfully used to rationalize the effects of chain elongation (Liljefors et al., 1985, 1987), change of double-bond configuration (Liljefors et al., 1987), and the introduction of an additional (*E*)-double bond at various positions (Bengtsson et al., 1987) on electrophysiological single-cell activities of analogs of (Z)-5-decenyl acetate (**1**), a sex pheromone component of the turnip moth, *Agrotis segetum* (Bestmann et al., 1978; Arn et al., 1980; Löfstedt et al., 1982).

Furthermore, the receptor–interaction model has been employed to predict the biologically active conformation of (Z)-5-decenyl acetate (Bengtsson et al., 1987). The predicted conformation is shown in Scheme 1. This prediction has recently been corroborated by the use of conformationally constrained analogs (Bengtsson, 1988).

The basic idea in our previous structure–activity studies is that, in order to show activity, it is necessary for an analog to mimic the natural pheromone component with respect to the spatial locations of the acetate group, the double-bond, and the terminal methyl group. It is assumed that the receptor cavity contains highly complementary interaction sites for these three molecular parts. All analogs previously studied were chosen for their ability to mimic the natural substrate in this respect through conformational rearrangements. We found that the observed electrophysiological activities for such analogs were determined essentially by the conformational energies required for the analogs to mimic the natural substrate.



SCHEME 1.

Since our receptor–interaction model at the present stage of its development requires mimicking of the acetate group, the double bond, and the terminal methyl group of the natural substrate, it cannot be applied to compounds that are unable to mimic the natural substrate in this respect. Thus, an important class of analogs that presently cannot be treated by our model is chain-shortened analogs. The acetate group, the double bond, and the terminal methyl group in this type of compound cannot simultaneously interact optimally with all three receptor sites of our model.

Chain shortening has consistently been found to lead to a decrease in the activity. However, for analogs one or two methylene units shorter than the natural component, a significant amount of the activity seems to be retained (Priesner et al., 1975; Priesner, 1979a,b, 1983; Struble and Byers, 1987).

Most of the previous studies on chain-shortened analogs have been performed with the EAG technique or by field-trapping experiments. Since chain-shortened analogs may be structurally very close to more than one of the components in the pheromone blend of the studied species, they may interact with more than one receptor type. Thus, the results of such studies are not suitable to be directly interpreted in terms of interactions with a single receptor type. In this respect, only single-cell recordings can provide unambiguous experimental data.

In order to further develop our receptor-interaction model, we have in the present paper studied a series of chain-shortened analogs, compounds **2–7**, of (*Z*)-5-decenyl acetate **1** (Figure 1). We report the synthesis of and electrophysiological single-cell measurements on them. The implications of the experimental data on the shape and other properties of the receptor cavity are discussed.

To corroborate the results, the accuracy of the stimulation system was investigated with respect to differences in volatility between test compounds and different amounts loaded onto the odor source.

METHODS AND MATERIALS

Chemicals. Final products were purified by flash chromatography (Taber, 1982a,b) on TLC-Silica gel 60 H supplied by Merck, and by argentation chromatography (Houx et al., 1974). All products were at least 98.5% pure and at least 99.7% pure with respect to geometric isomers as determined by capillary GLC on a Supelcowax 30-m or DB-Wax 30-m column.

Saturated acetates used for the methodological studies were from different sources, with the overall chemical purity always more than 95%.

Mass spectra were recorded on a Finnigan 4021 mass spectrometer, ^{1}H and ^{13}C NMR spectra on a Varian XL-300, a Nicolet 360 WB, or a Jeol FX-

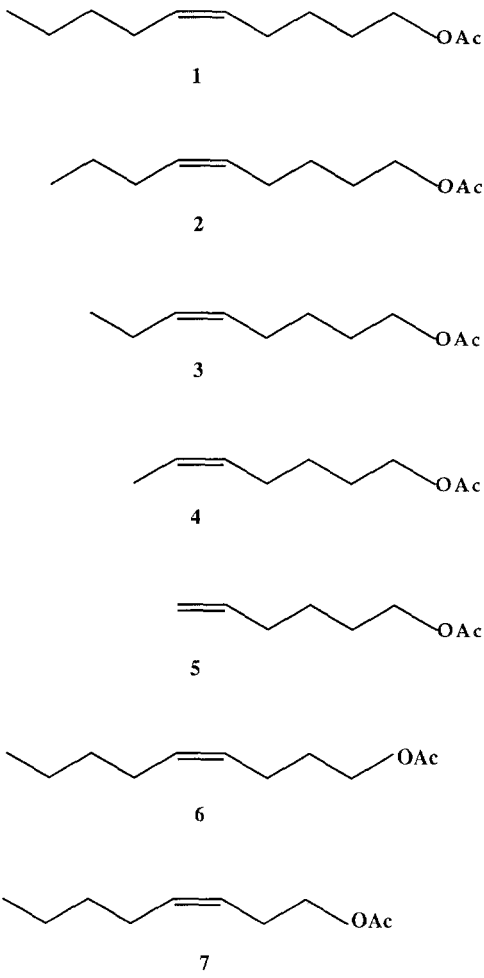


FIG. 1. Compounds studied.

60 spectrometer. NMR spectra were recorded on $CDCl_3$ solutions with Me_4Si as internal reference. For some of the products, the ^{13}C signal for the carbonyl carbon was of too low an intensity to be unambiguously identified.

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)-5-Decenyl Acetate (1). This was prepared as previously described (Olsson et al., 1983).

(Z)-5-Nonenyl Acetate (2). Ethyl 4-bromobutyrate was reduced with lith-

ium aluminium hydride, and the resulting bromoalcohol was protected with dihydropyran, according to standard procedures, affording 4-(2-tetrahydropyranyloxy)-1-bromobutane that was distilled at reduced pressure in base-washed glassware, bp 66–73°C/0.2 mm Hg. To a solution of liquid ammonia (100 ml) and dry ether (10 ml) at −78°C under a nitrogen atmosphere, lithium amide (0.92 g, 0.04 mol) was added in portions. The mixture was stirred for 20 min, and 1-pentyne (2.11 g, 0.03 mol) was added, according to a method described for similar systems by Berger and Canderday (1968). After stirring for an additional 45 min, 4-(2-tetrahydropyranyloxy)-1-bromobutane (5.00 g, 0.02 mol) dissolved in dry ether (25 ml) was slowly added. The resulting mixture was stirred for 2 hr at −78°C and then allowed to slowly warm up to room temperature while the ammonia evaporated. Water (50 ml) was added, and the resulting solution was extracted three times with hexane. The combined organic layers were washed with saturated NaCl solution and dried over $MgSO_4$. Removal of the solvent gave the crude product (4.04 g, 89%). Reduction with Lindlar catalyst (Lindlar, 1952; Maurer and Grieder, 1977; Leznoff et al., 1977; Marvell and Li, 1973; Wong et al., 1984), followed by acetylation (Schwartz and Waters, 1972) of the tetrahydropyranyl ether gave **2.** δ_H (300 MHz) 0.90 (t, 3H, Me), 1.31–1.46 (m, 4H, CH_2CH_2), 1.59–1.69 (m, 2H, $CH_2{-}C{-}O$), 1.96–2.10 (m, 4H, $CH_2C{=}$), 2.04 (s, 3H, Me−COO), 4.06 (t, 2H, $CH_2{-}OCO$), 5.34–5.40 (m, 2H, J_{AB} = 10.5 Hz, CH=CH). δ_C (75.4 MHz) 13.8, 20.9, 22.8, 26.0, 26.7, 28.2, 29.1, 64.5, 129.2, 130.3, 171.2. *m/z*: 124 (M^+-60; 7%), 109(1), 96(24), 81(35), 67(37), 61(2), 54(45), 43(100).

(Z)-5-Octenyl Acetate (3). 4-(2-Tetrahydropyranyloxy)-1-bromobutane (17.6 g, 0.074 mol) was reacted with lithium acetylide, ethylenediamine, complex, affording 14.4 g (62%) of 1-(2-tetrahydropyranyloxy)-5-hexyne (Smith and Beumel, 1974; Rossi et al., 1980; Jäger, 1977). Butyllithium (6 ml of a 1.44 M solution in hexane) was slowly added to 1-(2-tetrahydropyranyloxy)-5-hexyne (1.5 g, 8.2 mmol), dissolved in dry THF (8 ml). The solution was stirred at room temperature for 2 hr. Ethyl iodide (2.0 g, 0.013 mol) dissolved in DMPU (14 ml, freshly distilled from CaH_2) was added at such a rate that the temperature did not exceed 25°C (Bengtsson and Liljefors, 1988). The solution was stirred for 3 hr, then poured into ice water (50 ml) and extracted with hexane. The combined hexane layers were washed with saturated NaCl solution and dried over $MgSO_4$. After removal of the solvent, 1.69 g (92%) of crude 1-(2-tetrahydropyranyloxy)-5-octyne was obtained. After hydrogenation with Lindlar catalyst and acetylation, **3** was obtained. δ_H (300 MHz) 0.95 (t, 3H, Me), 1.38–1.46 (m, 2H, CH_2CH_2), 1.59–1.68 (m, 2H, $CH_2{-}C{-}O$), 2.01–2.10 (m, 4H, $CH_2C{=}$), 2.04 (s, 3H, Me−COO), 4.06 (t, 2H, $CH_2{-}OCO$), 5.28–5.41 (m, 2H, J_{AB} = 10.7 Hz, CH=CH). δ_C (75.4 MHz) 14.3, 20.5, 21.0, 26.0, 26.6, 28.1, 64.4, 128.4, 132.1, 171.2 *m/z*: 110 (M^+-60; 9%), 95(6), 82(40), 67(52), 61(2), 55(19), 43(100).

(Z)-5-Heptenyl Acetate (4) was prepared from 1-(2-tetrahydropyranyloxy)-5-hexyne and methyl iodide, according to the same procedure as described above for **3.** δ_H (300 MHz) 1.36–1.47 (m, 2H, CH_2CH_2), 1.58–1.69 (m, 5H, MeC=, CH_2—C—O), 2.03–2.11 (m, 2H, CH_2C=), 2.05 (s, 3H, Me—COO), 4.06 (t, 2H, CH_2—OCO), 5.32–5.52 (m, 2H, J_{AB} = 10.6 Hz, CH=CH). δ_C (75.4 MHz) 12.7, 21.0, 25.8, 26.3, 28.1, 64.5, 124.3, 130.0, 171.3. *m/z*: 96 (M^+-60; 11%), 81(20), 68(64), 61(3), 55(27), 43(100).

5-Hexenyl Acetate (5). 1-(2-Tetrahydropyranyloxy)-5-hexyne was reduced with disiamyl borane (Brown and Zweifel, 1961) and acetylated. δ_H (300 MHz) 1.37–1.48 (m, 2H, CH_2CH_2), 1.56–1.66 (m, 2H, CH_2—C—O), 2.00–2.09 (m, 2H, CH_2C=), 2.01 (s, 3H, Me—COO), 4.03 (t, 2H, CH_2—OCO), 4.91–5.02 (m, 2H, C=CH_2), 5.70–5.83 (m, 1H, CH=C). δ_C (75.4 MHz) 20.9, 25.1, 28.0, 33.2, 64.4, 114.8, 138.3, 171.2. *m/z*: 82 (M^+-60; 13%), 73(2), 67(29), 61(2), 54(35), 43(100). $C_8H_{14}O_2$ calc. C 67.6, H 9.9; found C 67.3, H 9.9.

(Z)-4-Nonenyl Acetate (6). 3-(2-Tetradropyranyloxy)-1-brompropane (prepared from 1-bromo-3-propanol and dihydropyran) was coupled with hexyne in liquid ammonia–ether as described for **2.** δ_H (300 MHz) 0.90 (t, 3H, Me), 1.28–1.35 (m, 4H, CH_2CH_2), 1.63–1.73 (m, 2H, CH_2—C—O), 1.99–2.15 (m, 4H, CH_2C=), 2.05 (s, 3H, Me—COO), 4.06 (t, 2H, CH_2—OCO), 5.31–5.43 (m, 2H, J_{AB} = 11.2 Hz, CH=CH). *m/z*: 124 (M^+-60; 9%), 109(1), 95(15), 81(38), 68(49), 61(1), 54(40), 43(100).

(Z)-3-Octenyl Acetate (7). 2-(2-Tetrahydropyranyloxy)-1-bromoethane (prepared from 1-bromoethanol and dihydropyran) was coupled with hexyne in liquid ammonia/ether as described for **2.** δ_H (300 MHz) 0.90 (t, 3H, Me), 1.30–1.36 (m, 4H, CH_2CH_2), 2.01–2.06 (m, 2H, CH_2C=), 2.04 (s, 3H, Me—CO), 2.36–2.41 (m, 2H, O—C—CH_2—C=), 4.06 (t, 2H, CH_2—OCO), 5.32–5.38 (m, 1H, J_{AB} = 11.2 Hz, CH=C). 5.46–5.52 (m, 1H, J_{AB} = 11.2 Hz, CH=C). *m/z*: 110 (M^+-60; 8%), 95(3), 81(17), 73(2), 68(20), 54(46), 43(100).

Electrophysiology. The biological activities of the pheromone component and of the analogs were investigated by the single sensillum technique (Kaissling, 1974), in which action potentials generated in olfactory sensilla on the male moth antenna were recorded. The method used was modified according to van der Pers and Den Otter (1978). Two days after emergence of the male moth, one of the antennae was excised and mounted in a grounded pipet electrode filled with Beadle-Ephrussi Ringer solution. To establish electrical contact with the olfactory neurons, the tip of a pheromone-sensitive sensillum was cut off. The Ringer-filled recording electrode was placed over the cut surface and connected to a high-impedance amplifier by an Ag–AgCl wire. The responses were stored on a Racal four-channel tape recorder and visualized on a storage oscilloscope.

The stimulus was applied to a piece of filter paper and put into a 5-ml plastic disposable syringe. Two milliliters of the syringe atmosphere was

injected by a hydraulic injection device (Murphy Developments, Hilversum, The Netherlands) into a purified and moistened air stream flushing over the preparation at a speed of 0.5 m/sec. For each stimulus concentration, 10 replicates were recorded.

Olfactory receptor cells specifically tuned to (*Z*)-5-decenyl acetate (**1**) are present in antennal sensilla type SW1 of *Agrotis segetum* and are readily accessible for single-cell recording (Hallberg, 1981; Löfstedt et al., 1982; van der Pers and Löfstedt, 1983). The receptor cell selective for (*Z*)-5-decenyl acetate could unambiguously be distinguished from the other receptor cells present in the sensillum by the amplitude of its action potential (Löfstedt et al., 1982). The responses of this receptor cell were recorded for all analogs.

The receptor cell response was defined by the number of action potentials generated during 1 sec from the onset of the stimulation. Dose–response curves were constructed using five different stimulus concentrations. For the pheromone component, **1**, the stimulus concentration ranged from 10^{-4} μg to 1 μg in decadic steps. For the analogs, the stimulus concentration ranged from 10^{-3} μg to 100 μg in decadic steps. The relative activity of each compound was expressed as the reciprocal of the relative quantities required to elicit the same response from the receptor cell, as the actual pheromone component elicited.

Stimulus Amounts Released from Odor Sources. The quantitative release of stimulus from the syringes used as odor sources (see above) was investigated by capillary gas chromatography on a Hewlett Packard 5830 GC equipped with a flame ionisation detector. Disposable syringes were loaded with stimulus applied to the filter paper in 100 μl of hexane. The syringe was fitted to an injection needle and 2 ml of the 5-ml syringe atmosphere was injected slowly (during approximately 1 min because of the limited flow capacity of the capillary column) splitless into the gas chromatograph. The initial temperature (60°C) was maintained for 2 min following the beginning of the injection. Then the split valve was opened, and the oven temperature was programmed at 5°C/min to 200°C. A 30-m × 0.25-mm-ID fused silica DB-1 column (J&W Scientific, Folsom, California 95630) was used for the separations. Hydrogen was supplied as carrier gas at 40 cm/sec linear velocity.

Six syringes containing a constant amount of the internal standard (*Z*)-5-decenyl acetate (500 μg) and different amounts of decyl acetate (30, 50, 100, 300, 500, and 1000 μg) were prepared to investigate the release of stimulus from odor sources loaded with different amounts of stimulus compound. The amount of decyl acetate relative to the internal standard was estimated in five consecutive injections for each dose. Absolute amounts released were estimated by comparison of peak areas with those obtained when known amounts of decyl acetate were injected split–splitless in 1–3 μl of hexane. In this experiment, the syringe atmosphere was allowed to equilibrate during the time of the preceding analysis (1 hr or more).

A mixture of six saturated acetates (hexyl, heptyl, octyl, nonyl, decyl, and undecyl) and the internal standard **1** was prepared to investigate the relation between volatility and relative amounts released from the odor sources. One hundred micrograms of each saturated acetate and 500 μg of the internal standard in 100 μl of hexane were applied to the odor source filter paper. In one experiment, the relative amounts released in five consecutive injections from the same syringe were investigated as described above. In a second experiment, the amounts released from five different, freshly prepared syringes were quantified. In this experiment, the hexane was allowed to evaporate, the syringe atmosphere were replaced once, and was then allowed to equilibrate during 2 min, before injection of the 2 ml on the GC.

RESULTS AND DISCUSSION

Dose–response curves in insect electrophysiology are usually based on amounts of stimuls applied to the odor source rather than the amount of stimulus to which the insect preparation is actually exposed. Quantification of the stimulus amounts released from the syringes used as odor sources in this and in our previous studies corroborated our assumption that the amounts released are proportional to the amounts applied to the filter paper (Figure 2) within the concentration range usually used in our experiments.

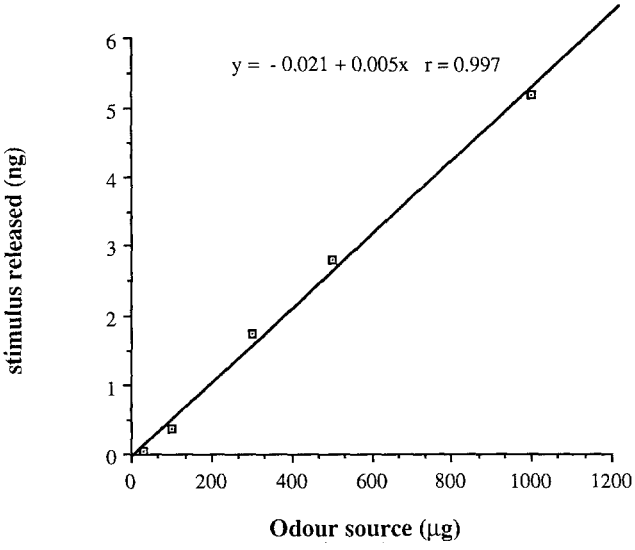


FIG. 2. Amounts of stimulus released from odor sources loaded with different amounts of decyl acetate on filter paper (N = 5).

The amount of a saturated acetate in 2 ml of syringe atmosphere was found to be proportional to the chain length of the acetate (Figure 3). The results of the two different experimental procedures shown in Figure 3A and B are essentially identical. The addition of one methylene unit on the average reduced the released amount by a factor of 3.9 [the average of exp (1.389) and exp (1.349)]. This factor is similar to the factor by which the saturated vapor pressures of straight-chain alkyl derivatives are reduced on the addition of a methylene group on the alkyl chain. Accurate vapor pressures for the entire series of alkyl acetates studied here are not available, but for lower homologs up to pentyl acetate,

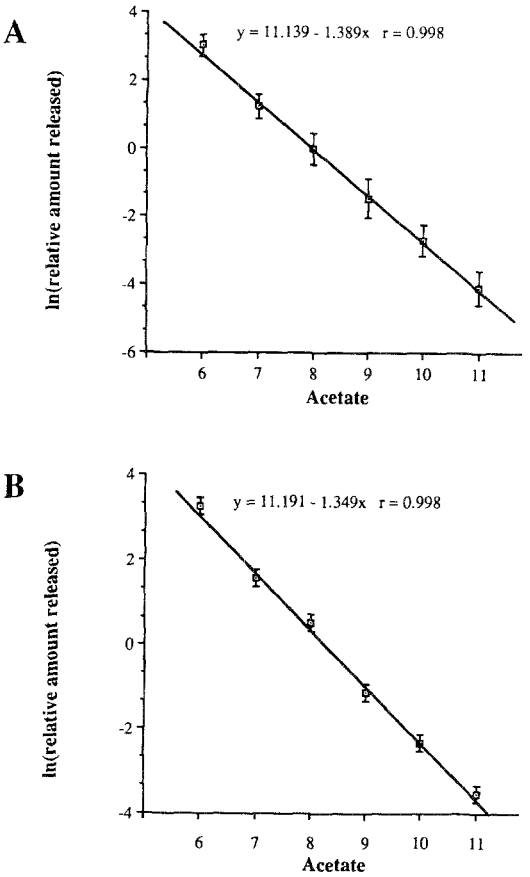


FIG. 3. Natural logarithm of relative amounts released of a homologous series of straight chain acetates (hexyl to undecyl acetate). The data points represent the average of injections from five consecutive injections from the same syringe (A) and five different freshly prepared syringes (B). (See text for further explanation.)

this factor is 2.8 (Jordan, 1954). The relative vapor pressures of decyl acetate (Olsson et al., 1983) and pentyl acetate correspond to a factor of 2.9 for each methylene group added to pentyl acetate. Thus, the relative amounts released from the odor source correspond to a reasonably good approximation to the relative saturated vapor pressures. The relative vapor pressures on chain shortening–chain elongation are similar for different classes of straight-chain alkyl derivatives. For a homologous series of alkanes (6–16 carbons), the corresponding factor is 3.2 (Stein, 1981). For straight-chain aldehydes (8–12 carbons) it is 3.7, and for 1-alkenes (10–13 carbons) it is 3.4 (Dykyj and Rep'as, 1979). Olsson et al. (1983) reported an average factor of 2.6 in the relative vapor pressures of monoenic acetates differing by one methylene unit. This indicates that relative standard vapor pressures may be used to estimate the relative amounts of compounds, differing in volatility, actually released from an odor source.

Our results demonstrate the necessity of corrections for differences in volatility when the electrophysiological activity of compounds with different chain lengths and/or vapor pressures are compared. For instance, with equal amounts of hexyl and decyl acetate at the odor source, approximately 231 times more hexyl acetate will stimulate the antenna if a chain length constant of 3.9 is used. Regardless of the exact value of the constant, corrected stimulus amounts are much more accurate than uncorrected ones.

The electrophysiological single-cell activities for compounds **1–7,** including corrections for volatility differences, are shown in Figure 4. All the chain-shortened analogs show a decrease of the biological activity compared to the natural pheromone component **1.** For the analogs shortened in the n-chain (Scheme 2), a loss of activity is found for each additional CH_2 group removed (compounds **2–5**). The loss of activity is about a factor of 16 for the removal of the first methylene group (compound **2**), and a factor of 6 for the removal of the second one (compound **3**). With further shortening of the *n* chain, the electrophysiological activity decreases drastically, finally giving the essentially inactive compound **5.**

These results are in line with those obtained by Priesner (1979a,b) in EAG screenings of several species of Noctuidae and Tortricidae. For five species, analogs chain shortened by one methylene unit showed activities 5.6–18 times less than those for the natural pheromone component. The analogs chain shortened by two and three methylene units were found to be 18–180 and 180–560 times less active, respectively. On stimulation of a male receptor cell of the

SCHEME 2.

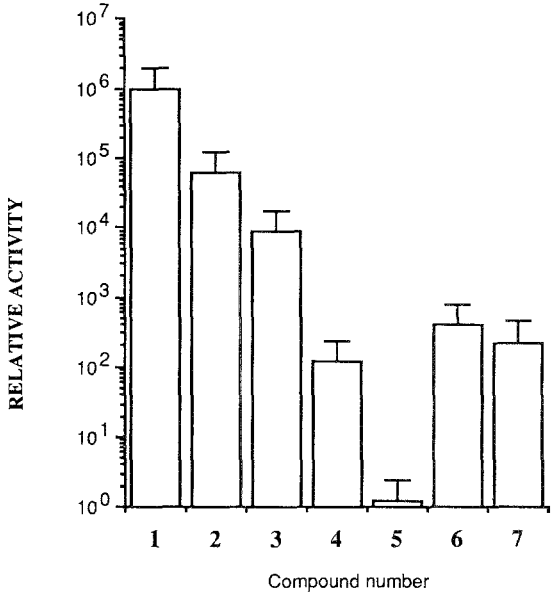


FIG. 4. Experimental single-cell activities for compounds **1–7.** The data are corrected for differences in vapor pressure. Error bars show maximal errors.

tortricid moth, *Adoxophyes orana*, selective for (Z)-9-tetradecenyl acetate, with (Z)-9-dodecenyl acetate, Priesner (1983) observed an activity loss of a factor of 30. Similarly, (Z)-7-decenyl acetate is about 25 times less active than the natural substrate on the (Z)-7-dodecenyl acetate receptor of *Agrotis segetum* (Liljefors et al., 1984). It should be noted that these data from the literature do not take differences in volatility into account.

The results shown in Figure 4 indicate that although the chain length of the natural component **1** is the optimal one, compounds one and two methylene units shorter can interact with and activate the receptor to a significant degree. For instance, compound **3** which is chain shortened by two methylene units, is 10 times as active as the corresponding compound chain elongated by two methylene units (Liljefors et al., 1987). The quite high activities of compounds **2** and **3** indicate that not only the terminal methyl group but also at least the next two methylene groups in the chain may efficiently interact with a hydrophobic region of the assumed receptor cavity, a region which is complementary to the van der Waals surface of the substrate molecule. Thus, the shape of the part of the receptor cavity interacting with the *n* chain may be a deep "groove" or "pocket," at least partly circumscribing the aliphatic chain. Figure 5 shows a clipped dot-surface representation of the van der Waals surface of part of the *n*

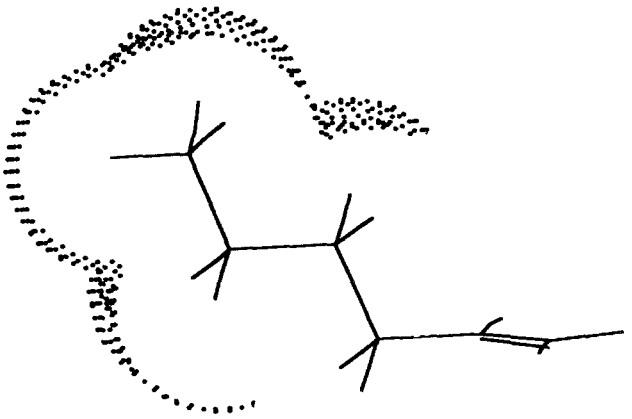


FIG. 5. A cross-section of the van der Waals surface of part of the terminal alkyl chain in compound **1**, indicating the size and shape of the complementary receptor cavity area interacting with this part of the molecule.

chain indicating a possible shape of this region of the receptor cavity. The lower part of the surface in Figure 5 is left undefined. It should correspond to a cavity large enough to accommodate the chain loops formed by chain-elongated analogs according to our receptor–interaction model (Liljefors et al., 1987).

From binding experiments on various enzymes, a maximum binding energy of 3.2 kcal/mol has been evaluated for a methyl group interacting with a site with "perfect" complementarity (Fersht, 1985). This value for hydrophobic binding of a methyl group is somewhat larger than that obtained from free energies of transfer of hydrocarbons from aqueous solution to pure liquid hydrocarbon, 2.1 kcal/mol (Tanford, 1980). Since the cavity in the enzyme is preformed, while the cavity in solution must be created to accommodate the solute, this is to be expected.

In order to make comparisons with binding energies possible, conformational entropies must be taken into account. The chain-shortened analogs **2–5** have one single bond less for each methylene group removed compared to the parent compound **1.** If we assume, as in previous studies (Liljefors et al., 1985, 1987; Bengtsson et al., 1987), that the molecule is rigidly bound to the receptor, the loss of conformational entropy on binding to the receptor is smaller for a chain-shortened analog of **1** than for **1.** Assuming an entropy loss of ca. 4 cal/mol K for "freezing" an internal rotation (Winnik, 1981; Liljefors et al., 1985), this difference may be estimated to be ca. 1 kcal/mol for each methylene unit removed. The maximum binding energy ("perfect" complementarity) for the terminal methyl of compound **1** then becomes $3.2 - 1.0 = 2.2$ kcal/mol.

Previously we found that an increase of the interaction energy of 1.6 kcal/

mol corresponds to an activity decrease by a factor of 10 (Liljefors et al., 1987; Bengtsson et al., 1987). As the activity drops by a factor of 16 on going from compound **1** to compound **2** (Figure 4), this corresponds, according to our model, to a loss of 1.9 kcal/mol of interaction energy. Note that relative activities reflect differences in total interaction energies. The total interaction energy is a sum of intermolecular interaction energies due to hydrophobic interaction, hydrogen bonding, and so on, subtracted by the conformational energy needed for the substrate molecule to acquire the "correct" conformation. In our previous work on compounds that have the ability to mimic the natural pheromone component, differences in total interaction energies only depend on the conformational energy term (Liljefors et al., 1987; Bengtsson et al., 1987). However, for chain-shortened analogs, differences in total interaction energies also depend on the intermolecular interaction energy term.

Shortening of compound **1** by one methylene group removes the methyl group from its interaction site (see Figure 5). Since the calculated energy loss is close to the value for maximum binding energy of a methyl group as derived above, this strongly indicates that the terminal methyl group is interacting with a very high degree of complementarity with its receptor site in the receptor cavity. This supports our model, in which the terminal methyl group of the natural pheromone component **1** is assumed to interact with a very well-defined receptor site (Liljefors et al., 1987).

In our receptor–interaction model, the double bond is also assumed to interact with a well-defined receptor site. This implies that the terminal methyl group in compound **2** cannot interact with the methyl group site in the receptor cavity—the *n* chain is too short. Providing that the "pocket" that binds the *n* chain extends to the methylene unit in the 8-position, shortening of the *n* chain in **2** by one methylene group to give compound **3** should result in a somewhat smaller activity loss than for the corresponding shortening of compound **1.** The decrease in activity should correspond to the loss of hydrophobic interactions of one methylene group, which in energy terms is about one third of the hydrophobic binding energy for a methyl group (Tanford, 1980). The experimental data support this conclusion: the activity drops by only a factor of 6 on going from compound **2** to compound **3** (Figure 4). On further shortening of the *n* chain (compounds **4** and **5**), the observed low activities (Figure 4) indicate that the alkyl groups have no interactions, or only very weak interactions, with the receptor.

When chain-shortening is done in the *m* chain (compounds **6** and **7,** Figure 4), the loss of electrophysiological activity is more dramatic. The removal of one or two CH_2 groups from the *m* chain results in a decrease of the biological activity by a factor of about 3000. From extensive EAG investigations of noctuid moths, Bestmann and Vostrowsky (1982) concluded that structural variations of the alkyl parts of a pheromone component of the investigated species

produce a more drastic loss of activity if the *n* chain is varied than when the equivalent changes are made in the *m* chain. This "structure–activity rule" is not in agreement with the results in Figure 4, and we have previously found that this "rule" is also not in agreement with experimental data for dienic analogs of (Z)-5-decenyl acetate (Bengtsson et al., 1987).

Interestingly, the experimental single-cell data (Figure 4) show that essentially the same loss of activity is obtained with the removal of one or two CH_2 units from the *m* chain. This was also observed by Priesner (1979a,b) in the EAG screening relative to above. Similar observations were done in a single-cell study on pheromone components of some saturniid species (Bestmann et al., 1987). In the single-cell study of *Adoxyphyes orana* mentioned above, Priesner (1983) found that a shortening of the *m* chain of (Z)-9- and (Z)-11-tetradecenyl acetate by two methylene units resulted in an activity decrease by factors of 100 and 30, respectively. When these values are corrected for differences in volatility, the first one is similar to the results we obtain for the corresponding compound **7.**

In our study on modifications of the acetate group and the corresponding effects on the electrophysiological activity (Liljefors et al., 1984), we found very strict requirements on the shape as well as on the electron distribution of the polar functional group for a productive interaction with the receptor. The results in Figure 4 are consistent with this conclusion. If the receptor cavity is highly complementary to the acetate group in **1,** as illustrated in Figure 6, a shortening of the *m* chain in this compound with one methylene group essentially destroys all interactions between the acetate group and the receptor. As shown in Figure 7, the orientation of the acetate group is very different in the

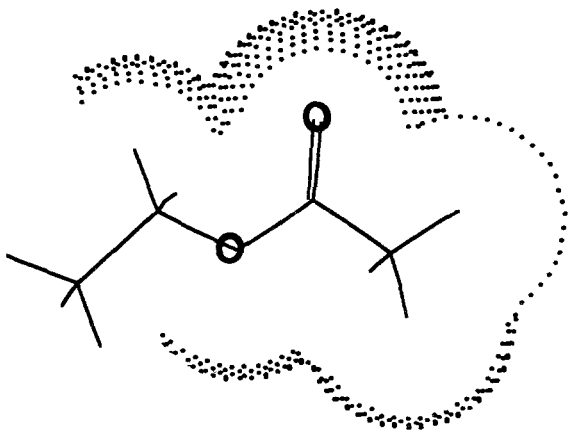


FIG. 6. A cross section of the van der Waals surface of part of the acetate group.

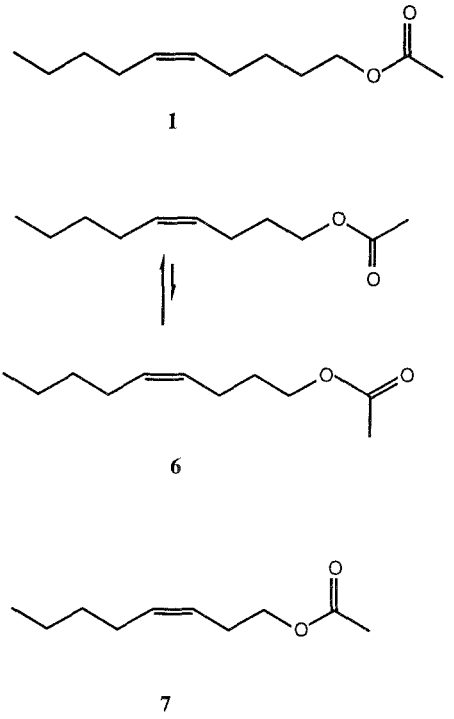


FIG. 7. Orientations of the acetate group in compounds **1**, **6**, and **7**.

thermodynamically preferred conformers of compounds **1** and **6**. We have found previously that the direction of the dipole moment of the polar functional group is of decisive importance for a productive receptor interaction (Liljefors et al., 1984). The direction of the dipole moment of the acetate group is approximately parallel to the carbonyl group. Thus, these dipole moment directions are antiparallel in **1** and **6**, which is incompatible with a productive receptor interaction for **6**. Rotation about the C—O bond in **6** (Figure 7) changes the dipole moment direction of its acetate group, but it is still far away from the optimal direction. Furthermore, such a conformational rearrangement is of prohibitively high energy, 8.5 ± 1.0 kcal/mol (Blom and Günthard, 1981).

Since the type of interaction between the acetate group in **1** and the receptor is not known, it is not possible to calculate the loss of interaction energy on going from compound **1** to **6**. However, as all parts of the acetate group seem to contribute to this interaction energy (Liljefors et al., 1984), this loss should be greater than the loss of interaction energy on removal of a methyl group from its site as discussed above.

In compound **7** the direction of the dipole moment of the acetate group is

the same as in **1** (Figure 7). However, an optimal interaction with the receptor sites by this group removes the double bond and the terminal methyl group from their sites. It is more likely that these interactions are as in **1** and that the acetate group only very weakly interacts with the receptor sites occupied by the acetate group in compound **1.** The experimental data in Figure 4 support this conclusion. Shortening the *m* chain in **6** to give compound **7** should, in this case, only reduce the activity by a factor corresponding to, at most, the loss of the hydrophobic interactions of one methylene group. As discussed above, for the *n* chain this corresponds to an activity drop by about a factor of 6. The observed activity for **7** is lower by a factor of about two compared to that for **6** (Figure 4), which is in reasonably good agreement with this prediction.

CONCLUSIONS

The terminal methyl group as well as the acetate group in the natural pheromone component **1** are concluded to interact with highly complementary sites in the receptor cavity. Thus, the dimensions of the receptor cavity in this respect correspond very closely to the length of the natural substrate. Our analysis of the experimental single-cell data indicates that the *n* chain binds to a hydrophobic "pocket" that extends over the two methylene groups closest to the terminal methyl group. Thus, compounds that are chain shortened by one or two methylene units have sufficiently strong interactions with the hydrophobic "pocket" to activate the receptor to a significant degree.

Chain shortening of one methylene unit in the *m* chain leads to a large drop in activity, probably due to the more or less complete removal of the strong interaction between the acetate group and its receptor sites. Further shortening of the *m* chain leads to a much lower activity loss due to loss of only hydrophobic interactions.

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CHEMICAL DEFENSE SECRETIONS OF SOME SPECIES OF MALAYSIAN RHINOTERMITIDAE (ISOPTERA, RHINOTERMITIDAE)

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Abstract—The defense secretions of the soldiers of the Malaysian rhinotermitid, *Parrhinotermes aequalis* (Havilandi) and *P. pygmaeus* (John), *Termitogeton planus* (Havilandi) and *Schedorhinotermes malaccensis* (Holmgren) consist mainly of vinyl ketones, whereas that of *Prorhinotermes flavus* (Bugnion & Popoff) gives (*E*)-1-nitropentadecene. The chemistry of the defense secretions of *Parrhinotermes* and *Termitogeton* is documented and based on their chemical relationships; *Termitogeton* shows a closer affinity to the Rhinotermitinae than Heterotermitinae.

Key Words—Isoptera, Rhinotermitidae, defense secretions, *Termitogeton*, *Parrhinotermes*, *Prorhinotermes*, *Schedorhinotermes*.

INTRODUCTION

The family Rhinotermitidae in Peninsular Malaysia is represented by six genera belonging to five subfamilies. The soldiers of all these genera have prominent and well-developed mandibles, which they use effectively for the defense of their colonies. In addition, copious amounts of chemicals are produced in their frontal glands that are used for defense. These chemicals are ejected via an opening (fontanelle) on the top of the head capsule during defensive action. The dimorphic soldiers of *Schedorhinotermes* and monomorphic soldiers of *Parrhinotermes* (Rhinotermitinae) have developed large tonguelike labra with a frill of short hairs at the distal end. These are used like brushes to spread the chem-

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ical secretions as they flow out from the fontanelle. None of the other genera exhibit this morphological adaptation. In *Prorhinotermes* (Prorhinotermitinae), a prominent groove leads away from the fontanelle. *Termitogeton* (Termitogetoninae) and *Heterotermes* (Heterotermitinae) have very small frontal gland openings. In contrast, *Coptotermes* (Coptotermitinae) has large and conspicuous fontanelles, and the frontal gland is prominent and well developed, extending well into the abdominal cavity of the soldier termites.

The chemical secretions of *Schedorhinotermes* and *Prorhinotermes* have been reported to consist mainly of a lipophilic mixture of electrophilic compounds such as vinyl ketones (*Schedorhinotermes*) and nitroolefins (*Prorhinotermes*), which act as contact poisons (Prestwich, 1979b). The daubing-brush method of application of the chemicals as practiced by *Parrhinotermes* and *Schedorhinotermes* must have undergone considerable morphological adaptions over time in order to arrive at its current efficacy (Prestwich, 1979a,b, Prestwich and Collins, 1982).

In this paper we report our investigations on the defense chemicals from Malaysian rhinotermitid species belonging to five genera and their chemical affinities.

Genus *Parrhinotermes* are small termites that feed on woody litter and are mostly found under the bark of trees forming distinct tracks and galleries in the wood they infest. They do not import soil into their feeding sites. The soldiers are monomorphic and are generally smaller than the worker caste. Chemical secretions are released through the frontal glands. Two species, *P. aequalis* (Havilandi) and *P. pygmaeus* (John), were investigated in this study.

Genus *Termitogeton* termites are found in logs of wood infested by wet and brown rot lying on the forest. The monomorphic soliders are flattened dorsoventrally, with legs displaced to the sides of the body. Defense secretions are released through the small frontal opening on the top of the head. Nests are generally found in proximity to the feeding sites. *Termitogeton planus* (Havilandi) is the only species found in Peninsular Malaysia.

Only one species of genus *Prorhinotermes*, *Prorhinotermes flavus* (Bugnion & Popoff), is so far known in Malaysia. This species is commonly found in mangrove swamps. Nests are built inside the stems of dead trees or in the tree stumps on the landward side of the mangrove. Their nests consist of many galleries in the wood. Defense secretions are released through the unmodified frontal gland pore of the monomorphic soldiers.

The genus *Schedorhinotermes* is found generally in closed canopy forests. The soldiers are dimorphic and have very well-developed labra. They nest in dead tree stumps and within hollowed trees. The species *Schedorhinotermes malaccensis* (Holmgren) feed primarily on wood undergoing initial rot. The major soldier is almost twice the size of the minor. Minor soldiers are in turn smaller than the workers. These termites forage under covered trails.

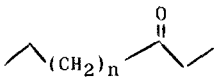
In the genus *Coptotermes* six species are reported in Malaysia. *Coptotermes curvignathus* is by far the most common species and can be found in natural forest, agricultural, and urban areas. The species is a recognized pest of forest and agricultural crops as well as timber and houses. The soldiers are monomorphic and can inflict a vicious bite when agitated. At the same time they release copious amount of a sticky white fluid. The genus is found mainly in tropical regions.

METHODS AND MATERIALS

Collection and Isolation. Part colonies of *Parrhinotermes aequalis*, *P. pygmaeus*, *Schedorhinotermes malaccensis*, *Termitogeton planus*, and *Coptotermes curvignathus* were obtained from Pasoh Forest Reserve in the state of Negri Sembilan; *Prorhinotermes flavus* was collected from Kuala Selangor forest reserve. The soldiers were removed from the colonies and stored in the freezer at −10°C overnight. Extractions of defense secretions from the crushed soldier heads (ca. 10) were carried out using hexane. The crude extracts were then filtered, and the solvent was partially removed in vacuo before analysis.

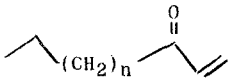
Analytical Method. Gas chromatography (GC) was performed on a HP 5790a instrument fitted with a flame ionization detector using a 2.1-m × 3-mm 3% OV-101 on 120–140 Gas Chrom Q column. Gas chromatography–mass spectrometry (GC-MS) was performed on a Pye 104 gas chromatograph fitted with the above column interfaced to a double-beam Kratos MS30 mass spectrometer by a membrane separator. Mass spectra were obtained at 70 eV with the MS source at 150°C and membrane interface temperature at 230°C. Mass spectral data were accumulated by a Kratos DS55 data system. The data on the vinyl ketones (Figure 1, **I–IX**) were characterized by GC, GC-MS, and coinjection with frozen and sealed extracts from *Schedorhinotermes lamanianus* Sjöstedt previously supplied by Prestwich for the identification of compounds **I–III** and **V–VII** (Prestwich et al., 1975). Compound **X** with molecular ions at *m/z* 255 was identified based on similar MS data reported for *Prorhinotermes simplex* by Vrkoc and Ubik (1974). The results (Table 1) indicate that the secretion from *Prorhinotermes flavus* is almost exclusively 1-nitropentadecene (**X**).

Three compounds not previously reported were identified as 1,14-pentadecadien-3-one (**IV**) from *Schedorhinotermes malaccensis* and 1,16-heptadecadien-3-one (**IX**) from *Termitogeton planus*, both being homologs of compounds **III** and **VII**. 1-Heptadecen-3-one (**VIII**) from *Parrhinotermes pygmaeus* was identified as a homolog of vinyl ketones **II** and **VI**. These three compounds, **IV**, **VIII**, and **IX**, are assigned 1,14-pentadecadien-3-one, 1-heptadecen-3-one, and 1,16-heptadecadien-3-one, respectively, based mainly on GC retention times and GC-MS spectral characteristics of expected homologs. The mass spectra of **IV**, **VIII**, and **IX** exhibit molecular ions at *m/z* 222, 252,



I n=7

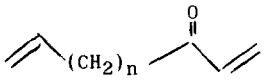
V n=11



II n=7

VI n=11

VIII n=12

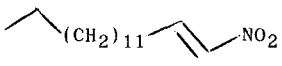


III n=9

IV n=10

VII n=11

IX n=12



X

FIG. 1. Defense compounds from Rhinotermitidae soliders.

TABLE 1. VINYL KETONE AND NITROOLEFIN COMPOSITIONS (%) FROM RHINOTERMITIDAE[a] SOLDIER SECRETIONS

Compound	Pp	Pa	S	Pr	T
Dodecan-3-one (**I**)	1	—	—	—	1
1-Dodecen-3-one (**II**)	2	—	—	—	—
1,13-Tetradecadien-3-one (**III**)	—	—	5	—	—
1,14-Pentadecadien-3-one (**IV**)	—	—	95	—	—
Hexadecan-3-one (**V**)	4	—	—	—	—
1-Hexadecen-3-one (**VI**)	82	99	—	—	94
1,15-Hexadecadien-3-one (**VII**)	5	1	—	—	2
1-Heptadecen-3-one (**VIII**)	6	—	—	—	2
1,16-Heptadecadien-3-one (**IX**)	—	—	—	—	1
1-Nitro-E-1-pentadecene (**X**)	—	—	—	99	—
Unknown	—	—	—	1	—

[a] Pp = *Parrhinotermes pygmaeus*; Pa = *Parrhinotermes aequalis*; S = *Schedorhinotermes malaccensis*; Pr = *Prorhinotermes flavus*; T = *Termitogeton planus*.

and 250, respectively, and show vinyl ketone fragments at *m/z* 55 of $CH_2{=}CHC{=}O^+$ and *m/z* 70 of $CH_2{=}CHC(OH)CH_2^+$.

RESULTS AND DISCUSSION

The family Rhinotermitidae from the Malaysian region is represented by five subfamilies: Termitogetoninae (Holmgren), Coptotermitinae (Holmgren), Prorhinotermitinae (Quennedey & Deligne), Heterotermitinae (Froggart), and Rhinotermitinae (Froggart). Psammotermitinae and Stylotermitinae are absent in this region (Roonwal, 1970). Previous work on Rhinotermitidae has shown that soldier termites from the subfamily Coptotermitinae, which includes the genus *Coptotermes*, produce mainly hydrocarbons and mucopolysaccharides of glucosamine (Moore, 1968: Prestwich, 1979a). The soldiers of *Coptotermes curvignathus* found in the Malaysian region are capable of releasing large droplets of similar white latexlike fluid through the large frontal openings on the head. This fluid is well suited to immobilizing small assailants because it dries quickly and entangles them. Current chemical analysis has shown that it is a suspension of saturated *n*-alkanes (C_{22}–C_{27}) in an aqueous solution of mucopolysaccharides of glucosamine and glucose units. Apart from chemical secretion, this termite also employs small, slender, incurved mandibles that are well adapted for piercing action.

The soldier of *Termitogeton planus* of the subfamily Termitogetoninae have a characteristic flat, heart-shaped, and hairy head from which they secrete mainly vinyl ketones (Table 1). These soldiers have well-developed slender, inwardly curved mandibles adapted for biting or piercing action with the simultaneous ejection of vinyl ketones into the wound of the predator.

The genus *Prorhinotermes* of the subfamily Prorhinotermitinae is represented by *P. flavus* in Malaysia. The soldier frontal gland of *Prorhinotermes* has a hypertrophied reservior cavity and is well adapted for ejection of defense secretions, especially against its predators such as ants. The genus *Prorhinotermes* was elevated to the current monogeneric subfamily Prophinotermitinae within the Rhinotermitidae because of different morphological features (Quennedy and Deligne, 1975). The secretion chemistry of the Malaysian *P. flavus*, in which nitroalkene **X** is produced, is also different from the usual vinyl ketones of Rhinotermitinae. The long-chain nitroalkene **X** has also been reported previously for *Prorhinotermes simplex* (Hagen) from Cuba (Vrkoc and Ubik, 1974). Nitroalkenes would therefore appear to be a taxonomic feature for soldiers of *Prorhinotermes*.

The frontal glands of major and minor soldiers of *Schedorhinotermes* are well adapted for daubing-brush chemical defense. Several *Schedorhinotermes* species have been reported to release odorous repellents consisting of vinyl

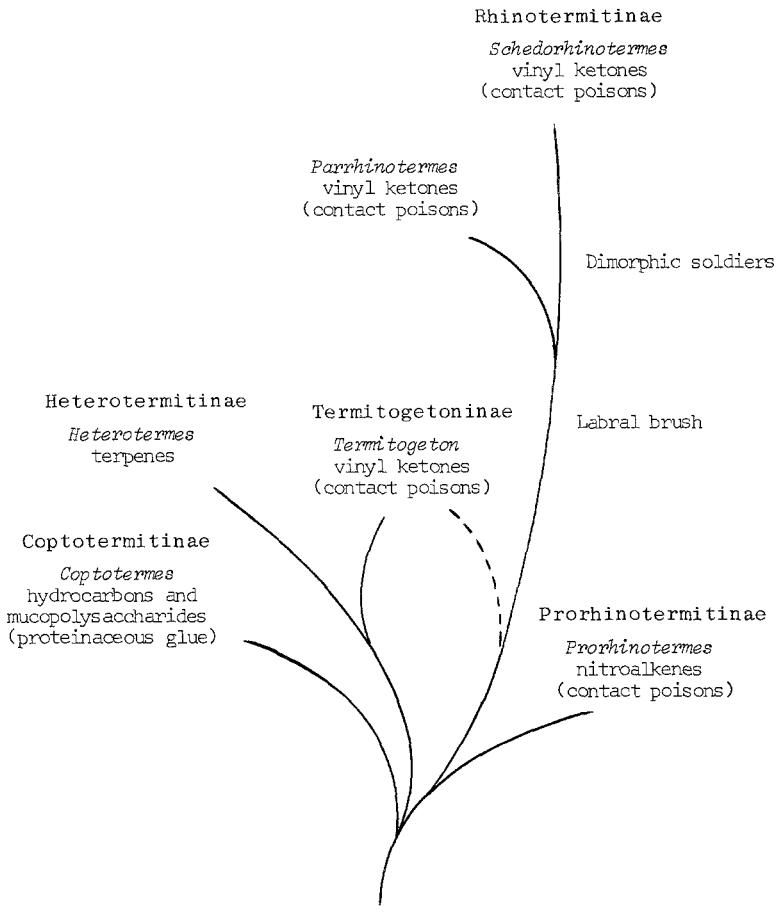


FIG. 2. Phylogeny of Rhinotermitidae—redrawn from Quennedey and Deligne (1975) with modification based on present study.

ketones. Several alkanones, alkenones, and alkadienones have been identified and are known to have a toxic effect on ants (Quennedey et al., 1973; Prestwich et al., 1975). The Malaysian *Schedorhinotermes* and *Parrhinotermes* of the subfamily Rhinotermitinae are now shown to produce mainly vinyl ketones (Table 1). *Parrhinotermes* is of interest since it has been considered the most primitive genus of Rhinotermitinae family (Quennedey and Deligne, 1975). Morphological comparisons of the soldiers of *Schedorhinotermes* and *Parrhinotermes* (both have well-developed labra with "brushes" on the distal ends) would already suggest the defense chemicals of the two genera would not be

too different from each other. Indeed, the chemicals secreted by *P. pygmeaus* and *P. aequalis* consist of a mixture of vinyl ketones, the dominant one being 1-hexadecen-3-one (**VI**) as shown in Table 1. Thus, the similarity of the chemistry of their defense secretions supports their close phylogenetic position as already evident based on their morphology (Prestwich, 1983).

It should be noted that defensive secretions from other *Schedorhinotermes* spp. from different parts of the world (Moore, 1968; Prestwich et al., 1975; Prestwich and Collins, 1982; Quennedey et al., 1973) also consistently have been determined to consist of vinyl ketones. Based on their studies, Quenneday and Deligne (1975) had proposed the phylogenetic relationships for the various genera and subfamilies in the Rhinotermitidae (Figure 2). In their interpretation of the phylogenetic tree, they have placed the subfamily Termitogetoninae together with Heterotermitinae on a line distinctly separate from the Rhinotermitinae and *Prorhinotermitinae*. However, as evident from this study, the soldier defense secretions of *Termitogeton* have been shown to consist of mainly vinyl ketones, as occur in *Parrhinotermes* and *Schedorhinotermes*. This would suggest that the subfamily Termitogetoninae could be much more closely related to the Rhinotermitinae than Heterotermitinae. Hence it should be more appropriate to place it on the same phylogenetic line (this is indicated in Figure 2 by broken lines).

It has also become quite evident from current knowledge of the chemistry of the defense secretions of the family Rhinotermitidae that they are all derivatives of polyketide/fatty acid biosynthesis, therefore providing a common link and possibly common ancestral stock. Hence, for this family, the secretion chemistry provides useful and vital information for the taxonomic and phylogenetic positions of the various genera and subfamilies.

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DERMATITIS-INDUCING FURANOCOUMARINS ON LEAF SURFACES OF EIGHT SPECIES OF RUTACEOUS AND UMBELLIFEROUS PLANTS[1]

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Abstract—Eight species of Rutaceae or Umbelliferae, known to cause or suspected of causing photophytodermatitis, had the linear furanocoumarins psoralen, bergapten, and xanthotoxin on their leaf surfaces, in concentrations varying from 0.014 to 1800 μg/g fresh weight, equivalent to 0.17–56% of the total leaf concentration. The higher percentage generally observed for spring leaves compared to autumn leaves suggests a higher rate of transfer of these furanocoumarins to the surface in the younger leaves. Among the plants studied, *Ruta graveolens* had the highest surface concentrations of all three furanocoumarins. The relatively high effectiveness in causing dermatitis of some species with low surface concentrations may be explained by a more effective mechanism of transfer of the furanocoumarins to the skin. A role in the defense of the plant is suggested by their accumulation on the plant surface.

Key Words—Furanocoumarins, plant surface, dermatitis, Rutaceae, Umbelliferae.

INTRODUCTION

Although the existence of furanocoumarins in many species of the Rutaceae and Umbelliferae is well documented (Gray and Waterman, 1978; Murray et al., 1982; Matern et al., 1988), attempts to localize them in these plants have been

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confined to entire organs, with very little attention to specific tissues (Andon and Denisova, 1974; Capalletti et al., 1984). In this laboratory, *Ruta graveolens* L. recently has been the subject of more detailed study in this context (Zobel and Brown, 1988, 1989). This species has long been notorious for inducing contact dermatitis (photophytodermatitis), which arises from the reaction of its linear furanocoumarins (psoralens, **1**) with skin DNA in the presence of UV radiation (Mitchell and Rook, 1979). High surface concentrations of these physiologically active coumarins (Zobel and Brown, 1989) can account for the marked propensity of *R. graveolens* to induce photophytodermatitis.

In the work reported here we have examined other species of the Rutaceae and Umbelliferae known to cause dermatitis (Mitchell and Rook, 1979) or suspected from anecdotal evidence of doing so, with the object of determining to what extent a substantial surface concentration of the three principal dermatitis-inducing psoralens is a general phenomenon in these families.

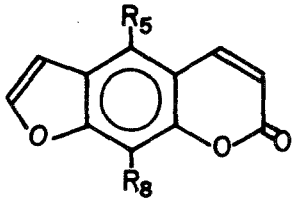
METHODS AND MATERIALS

R. graveolens was cultivated in a garden or greenhouse at Trent University, and *Heracleum lanatum* was collected from the wild state on the campus. The remaining species studied were obtained from the collection of the Royal Botanical Gardens, Hamilton, Ontario.

The procedure for removal of surface deposits by a single brief dipping of the leaves into almost boiling water (ca. 96°C, 100 ml) has recently been described (Zobel and Brown, 1988). In each case duplicate samples of ca. 10 leaves were used. Preliminary examination of the extracts was done by thin-layer chromatography on silica chromatoplates (Macherey Nagel, 0.25 mm thick) developed in ethyl acetate–isooctane, 1:3. This was followed by quantitative analysis of psoralens by high-performance liquid chromatography (Thompson and Brown, 1984; Zobel and Brown, 1989) on a 7.5-cm reversed-phase, 4-μm C_{18} column (Waters Nova-Pak) developed with 25% aqueous acetonitrile. Quantitation was by measurement of peak areas and reference to standard curves prepared from reference samples in our collection.

RESULTS

The three linear furanocoumarins predominating in these two families—psoralen (Scheme 1, **a**), bergapten (**b**) and xanthotoxin (**c**)—were detected on the surfaces of all five species of the Rutaceae and all three of the Umbelliferae examined in the present study (Table 1, values for duplicate samples shown in



1

a $R_5 = R_8 = H$

b $R_5 = OMe$, $R_8 = H$

c $R_5 = H$, $R_8 = OMe$

SCHEME 1.

each case). However, there was a great variation in the total surface concentrations, amounting to a range of five orders of magnitude, among the different species. They were highest on young spring leaves of *R. graveolens* (ca. 1800 μg/g fresh weight) and lowest on old, autumn leaves of *Evodia danielli* (0.014). In these two plants, as well as in *Orixa japonica* and *Peucedanum officinale*, the total concentrations of the three coumarins were higher on young leaves than on old, but the converse was the case in *Phellodendron chinensis*. Owing to unavailability of material at one or the other season, we could not compare the concentrations in the spring and autumn leaves of three species. The sum of the surface concentrations on spring leaves ranged from 6.7 to 56% of the total in the leaf, and on autumn leaves from 0.17 to 37%.

The three furanocoumarins in the species investigated were found in varying amounts on the leaf surface, with xanthotoxin predominant in many cases (Table 2). The concentration of this coumarin was highest on spring leaves of *R. graveolens* (ca. 880 μg/g fresh weight), with the next highest, *O. japonica*, having ca. 100, followed by *H. lanatum* with only ca. 0.5, differences of >1000-fold. Differences of an even greater order, >10,000-fold, were observed for psoralen, with respective values of 690, 1.6, and 0.06. Bergapten was again found in the highest concentrations on *R. graveolens* (220 μg/g fresh weight), followed by *O. japonica* with 22 and *Peucedanum officinale* with 0.56, a difference of ca. 400-fold.

In only one case (Table 3) were equivalent concentrations of the three

TABLE 1. CONCENTRATIONS OF FURANOCOUMARINS ON LEAF SURFACES OF RUTACEOUS AND UMBELLIFEROUS PLANTS AS PERCENTAGE OF TOTAL LEAF CONCENTRATION

Species	Leaves, autumn 1987		Leaves, spring 1988	
	Surface conc[a]	Average % of total in leaf	Surface conc[a]	Average % of total in leaf
Ruta graveolens	705, 740	37	1850, 1800	56
Orixa japonica	18.4, 19.5	2.8	120, 128	32
Phellodendron chinesis	0.076, 0.086	3.2	0.03, 0.03	17
Evodia danielli	0.018, 0.014	9.8	0.018, 0.024	8.0
Dictamnus albus	0.020, 0.030	0.17	NA	NA
Peucedanum officinale	0.099, 0.093	1.1	0.72, 0.60	6.7
Heracleum mangazzianianum	3.8, 34.	1.9	NA	NA
Heracleum lanatum	NA[b]	NA	0.71, 0.73	8.2

[a] μg/g fresh weight.
[b] NA, no sample available.

TABLE 2. CONCENTRATION OF PSORALEN, XANTHOTOXIN, AND BERGAPTEN ON PLANT SURFACE, AND PERCENTAGE OF EACH OF TOTAL CONCENTRATION IN LEAF

Species	Psoralen		Xanthotoxin		Bergapten	
	On surface[a]	% of total[b]	On surface[a]	% of total	On surface[a]	% of total
Autumn plants						
Ruta graveolens	230, 215	33.5	435, 450	39	55, 59	33
Orixa japonica	traces	NA[c]	14.9, 14.5	3.0	4.3, 3.9	2.5
Phellodendron chinensis	0.009, 0.009	0.98	0.034, 0.030	15.6	0.039, 0.035	2.9
Evodia danielli	0.008, 0.005	8.2	0.007, 0.007	10	0.003, 0.004	20
Dictamnus albus	traces	NA	0.011, 0.010	0.13	0.03, 0.02	0.3
Peucedanum officinale	0.008, 0.007	3.0	0.070, 0.053	1.7	0.028, 0.026	0.55
Heracleum magazzianianum	1.25, 1.20	2.0	2.2, 2.05	1.9	0.28, 0.28	1.5
Spring plants						
Ruta graveolens	750, 630	54	820, 940	56	200, 285	49
Orixa japonica	1.55, 1.70	22	88, 112	31	25, 19.5	39
Phellodendron chinensis	0.01, 0.01	10	0.008, 0.015	15	<0.01	ca. 14
Evodia danielli	<0.01, <0.01	ca. 50	0.01, 0.01	5	<0.01	ca. 21
Peucedanum officinale	0.01, 0.01	2	0.09, 0.07	7	0.50, 0.63	6.6
Heracleum lanantum	0.07, 0.05	9	0.55, 0.41	7.8	0.20, 0.16	9.4

[a] μg/g fresh weight.
[b] Average value.
[c] NA, no sample available.

TABLE 3. RATIOS OF CONCENTRATIONS OF PSORALEN, BERGAPTEN, AND XANTHOTOXIN ON PLANT LEAF SURFACES[a]

Species	Spring leaves P : X : B			Autumn leaves P : X : B		
Ruta graveolens	2.9	3.7	1	3.9	7.7	1
Evodia danielli	1	1	1	2.0	2.0	1
Phellodendron chinensis	3.0	1	1	0.23	0.82	1
Orixa japonica	1.2	0.02	1	0.001	3.6	1
Dictamnus albus				<0.001	1	1
Heracleum magnazzianianum	4.3	7.5	1			
Heracleum lanatum				0.33	2.7	1
Peucedanum officinale	0.29	2.2	1	0.018	0.14	1

[a]P, psoralen; X, xanthotoxin; B, bergapten.

coumarins observed, i.e., young *E. danielli* leaves. This table shows the proportions of the three, with that of bergapten taken as unity. Bergapten in the spring leaves appeared to be present in low concentrations, but in old leaves it can even predominate, as seen in *Peucedanum officinale* and *Phellodendron chinensis*.

Table 2 shows, as well as the concentration in micrograms per gram fresh weight, the concentration of each surface furanocoumarin as the percentage of its total concentration in the entire leaf. The higher percentage values were observed with spring leaves except for *Phellodendron chinensis*, where the xanthotoxin values for spring and autumn leaves were similar; *E. danielli*, where on autumn leaves the xanthotoxin values were higher; and in *Peucedanum officinale*, where the percentage of psoralen on autumn leaves was, if anything, higher than on the spring leaves. Data from Table 2 permit the inference that even the small concentrations of each coumarin on the surface are a substantial part of the total in the plant. The values in the upper part of the observed range of 0.13–56% indicate that a large part of the furanocoumarins produced within the cells is sent to the surface.

DISCUSSION

Furanocoumarins have long been known to exist in leaves of the species of Rutaceae and Umbelliferae examined here (Murray et al., 1982), but their localization had not been previously studied. In this work we have located psoralen, xanthotoxin, and bergapten on the surface of the leaves, to a substantial degree in many cases, and not only inside.

In the case of waxes, leaves can be washed with organic solvents for 10–

45 sec (Tulloch 1987; Tulloch and Hoffman, 1982) without concern about leakage, as these substances are known to exist on the surface only. However, for removal of furanocoumarins from *R. graveolens* leaves, we found organic solvents unsuitable (Zobel and Brown, 1989); methanol, for example, which is used for washing out flavonoids (Proksch et al., 1986), is well known as a tissue fixer that rapidly penetrates and denatures proteins, "fixing" the membranes. Concerned about such drastic action of methanol and acetone, we compared their ability to remove furanocoumarins with that of a very short extraction into almost boiling water. Amounts released into this last solvent were of the order of hundreds of times as high, or in some cases even higher, without leakage from the epidermal cells (Zobel and Brown, 1988), and corresponded in the present experiments to surface concentrations ranging from 0.17 to 56% of the total concentrations in the leaf. These values may still represent only minima—the smallest amounts obtainable after a single brief dipping into hot water, which at least partially removes an overcuticular layer. Further experiments are in progress to determine whether a longer dipping time will remove yet more material without leakage from the epidermal cells.

The total furanocoumarin concentration (psoralen + xanthotoxin + bergapten) differs in different species. *R. graveolens* has a remarkably high furanocoumarin concentration on the surface, even compared to the 28% of the total flavonoid concentration on the leaf surface of *Flourensia resinosa* (Wollenweber, 1986). As no mechanism for the synthesis of coumarins can exist on the surface, concentrations of all three furanocoumarins ranging between 700 and 1800 μg/g fresh weight on the surface indicate that this plant must export large amounts from the interior. The sum of the surface concentrations in *R. graveolens* was higher than that in the whole leaf of *Orixa*, which contains the next highest concentrations. Those of the three individual furanocoumarins are not equal (Table 3): xanthotoxin predominates in many cases, except for *Phellodendron* and *Peucedanum* (autumn leaves), where there is more bergapten, and in *Phellodendron* and *Orixa* (spring leaves), where the psoralen concentration is highest. The variation of proportions in autumn and spring leaves suggests developmental changes, and these variations are now being investigated in *R. graveolens*, *R. chalepensis*, and *H. lanatum* over more extended periods.

A point of importance is that even the small concentrations of furanocoumarins on the leaf surface, both individually and in total, constitute a significant percentage of the whole concentration of the plant leaf. This suggests active transport of these substances to the surface, where, as we have already discussed for *R. graveolens* (Zobel and Brown, 1989), they could play a defense role, forming a coating over the cuticular layer (see also the discussion below). Xanthotoxin is recognized as a plant toxicant (Berenbaum and Neal, 1985).

A further question of interest is why plants causing dermatitis do not contain approximately the same concentrations of furanocoumarins on the surface.

When two of the plants causing photophytodermatitis, *R. graveolens* and *H. mangazzanianum*, are compared, we note that the surface concentrations of furanocoumarins are drastically different: 1800 and 3.6 μg/g, respectively. The phenomenon could be at least partially explained by postulating different mechanisms of transferring these toxins to the skin. *R. graveolens*, having a smooth, hairless surface, could induce dermatitis when the grayish waxy cover is removed by touching. In contrast, *H. mangazzianianum* possesses erect trichomes with a very thick, strong cell wall and sharp tips that could mechanically penetrate the upper layer of the skin and carry their furanocoumarins beneath the surface. In this way smaller amounts on the surface of the plant could produce comparable levels of irritation.

Dictamnus albus seem to be more of a threat after the leaves have been crushed or when there are breaks in the skin. More studies are needed to determine if *D. albus* can induce contact dermatitis and if, at the concentration there (0.025 μg/g), it has an antibiotic function. In our laboratory, furanocoumarins in concentrations found on *R. graveolens* leaves (and even lower by a factor of 10) have been shown to play an antibacterial role, and they decreased mitoses in root tips of *Allium cepa* (A.M. Zobel and K. Mwiraria, unpublished).

In our experiment, substances other than the three furanocoumarins, exhibiting a blue-green fluorescence, were also extracted into water. As demonstrated by Cesca et al. (1986) in the case of *Daucus carota*, such a complex of various surface substances, including a furananocoumarin, can act as an insect attractant. We consider it possible that such complexes of surface compounds could serve as a defense barrier for the plant, not only at the actual surface but comparable on a microscale to the atmosphere around the surface of the Earth. For example, as furanocoumarins are readily sublimable, slow continuous sublimation under natural conditions could give such an atmosphere adjacent to the plant surface that is toxic to approaching bacteria or fungal spores. In this context we hope that workers elsewhere will be interested in identification of other compounds extracted from plant surfaces by our procedure.

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SUNFLOWER AROMA DETECTION BY THE HONEYBEE
Study by Coupling Gas Chromatography and Electroantennography

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Abstract—Combined electrophysiological recordings (EAG) and gas chromatographic separation were performed in order to investigate which volatile chemical components of a sunflower extract could be detected by honeybee workers and thus are likely to trigger the foraging behavior. A direct coupling device allowed for the stimulation of the antennal receptors with individual constituents of a polar fraction of the flower aroma shown to be attractive to bees. More than 100 compounds were separated from the extract. Twenty-four compounds elicited clear EAG responses. These compounds were identified by mass spectrometry (electronic impact and chemical ionisation). Both short- and long-chain aliphatic alcohols, one short-chain aliphatic aldehyde, one acid, two esters, and terpenic compounds were found to stimulate the antennal receptors. Six compounds identified in previous behavioral experiments were found to exhibit EAG activity. The chemicals screened by this method may be used for recognition of the plant odor and the selective behavior of honeybees.

Key Words—Insect–plant relationships, olfaction, honeybee, Hymenoptera, Apidae, sunflower, allelochemicals, coupling GC-EAG.

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INTRODUCTION

Chemical analyses of plant volatile extracts of various species showed a large diversity among different chemical classes. More than 50 compounds were found in artichokes (MacLeod et al., 1982), cotton (Hedin, 1976), Ranunculaceae (Pellmyr et al., 1984), maize (Buttery et al., 1978), about 150 in orchid flowers (Borg-Karlson and Tengo, 1986), and between 80 and 250 in different genotypes of sunflower (Etiévant et al., 1984; Pham Delègue et al., 1988). Moreover, these complex blends can fluctuate in quality and quantity according to plant phenology (Maarse and Kepner, 1970; Thompson et al., 1971; Hedin, 1976; Pham Delègue et al., 1988, Hamilton-Kemp et al., 1988), and also in response to climatic factors and soil characteristics (Robacker et al., 1982). One question arising is how phytophagous insects process the chemical information available and adapt their behavior to such complexity and variation of plant aromas (Masson, 1983).

In natural conditions, honeybee foragers are attracted to sunflower crops and are able to distinguish between different genotypes (Parker, 1981; Pham-Delègue et al., 1985). In a previous work, Pham-Delègue et al. (1986) conditioned bees to a sunflower extract and offered them a choice between the conditioning scent and different fractions of this extract. This work established the attractiveness of a fraction of the sunflower extract containing mainly the polar constituents and led to the identification of a limited number of molecules possibly involved in the recognition of the total aroma by bees. Electroantennogram responses (EAG) at the outlet of a gas chromatograph (GC) were recorded in order to determine which compounds of the polar fraction are detected by the olfactory receptor cells and hence are likely to be used as chemical cues to elicit the orientation of bees.

Most of the GC-EAG methods described in the literature for screening the olfactory activity of chemical substances are based on a trapping of these products at the outlet of the GC and a subsequent flushing of the trapped material onto the biological preparation (Moorhouse, 1969; Wallbank and Wheatley, 1979; Löfstedt et al., 1983). Such a technique is not practical when large numbers of constituents from plant extracts have to be tested for EAG activity. We therefore used a direct coupling of EAG and GC based on the same principles as those developed by Arn et al. (1975) and Wadhams (1982) for pheromones or by Guerin and Stadler (1984) for plant odors.

METHODS AND MATERIALS

Plant Extract. Ten sunflower heads were extracted in a Soxhlet apparatus with dichloromethane. Volatile constituents were removed from the resultant concentration by a high-vacuum cold-finger distillation. Volatile materials con-

tained in the cold traps and condensed on the cold finger were taken up in dichloromethane and concentrated. The extract was then separated into polar and apolar fractions by column chromatography on silica gel. The column was eluted with purified pentane (apolar fraction) and purified ether (polar fraction). The polar fraction was stored under nitrogen in sealed glass tubes at −20°C. The entire procedure was described elsewhere (Etiévant et al., 1984).

Insect Material. Worker honeybees (*Apis mellifera ligustica*, L.) emerging in June were kept in an incubator at 33°C and were fed with a piece of candy sugar and water. Electrophysiological recordings were performed when the bees were 8 days old (number of bees: 10).

Direct Coupling between Gas Chromatography and Electroantennographic Apparatus. Gas chromatography (GC) was performed on a Girdel 3000 gas chromatograph equipped with a J&W retrofit on-column injector (Jennings and Takeoka, 1984). A 30-m long fused silica megabore column (0.53 mm ID, J&W DB5, thickness 1.5 μm) was used for the separations. A deactivated fused silica column (5m) was connected to the megabore column as a retention gap (Grob et al., 1985). Helium was used as carrier gas with a velocity of 28 cm/sec. Samples of 0.4 μl were injected at room temperature into the precolumn, which was then pushed inside the oven. After 3 min at 40°C, the temperature was linearly raised at 3°C/min up to 250°C. At the exit of the column, the effluent was split into two parts: one directed onto the insect antenna (2/3), the other sent to the flame ionization detector (1/3). The splitter was a Y-shaped vitreous silica outlet splitter (SGE Ltd.) without gas makeup added. To avoid variations of the split ratio with temperature, the splitter and the two silica lines were maintained at a constant temperature of 250°C.

The molecules eluted from the column were blown onto the antenna in a humidified stream of purified air (speed 20 cm/sec, internal diameter of the glass tubing = 0.8 cm). The distance between the outlet of the silica line and the outlet of the tubing was set at 3 cm (ca. 4 cm from the antenna).

The relative concentration of each stimulating compound was expressed as a percentage of the sum of all the peaks after subtraction of the solvent peak area.

Electroantennograms (EAGs) were recorded by an electrode covering the cut tip of the left antenna of each bee. The indifferent electrode was gently inserted at the base of the same antenna. The bees were maintained inside a Perspex block adjusted to the bee. Glass electrodes were filled with a physiological saline solution (NaCl, 6.5 g/liter; KCl, 0.25 g/liter; $CaCl_2$, 0.3 g/liter; Na_2CO_3, 0.2 g/liter). An Ag–AgCl wire inserted into each electrode connected the antenna with the recording device: input probe, preamplifier (Carrack PB 181-11), and differential oscilloscope (Tektronix 5A22N). EAG amplitudes were measured on the oscilloscope screen and expressed in millivolts. The impedance of the preparations used was lower than 2×10^6 Ω. Changes in the sensitivity of the preparation were checked regularly with a

standard stimulus (1 ml of air flushed into the air stream through a pipet containing a filter paper soaked with 20 μl of hexanol in paraffin oil, dilution 1% v/v). Olfactory stimulations by the standard were delivered at least four times during each GC run. Compounds eliciting responses in more than eight of 10 preparations were considered as effective stimuli, others were ignored. Since the physiological significance of hyperpolarizing EAGs is questionable, the mean value of EAGs was calculated on the basis of depolarizations exclusively; confidence intervals (CI) are calculated as follows: $t \times (SD)/\sqrt{n}$ where SD is the standard deviation and t is the value of the t test at $P < 0.05$.

Chemical identifications of peaks eliciting significant EAG responses were performed using a coupled GC-MS device (GC Girdel 300, R 10/10 Nermag). Identifications were based on electron-impact ionization (EI) (70 eV). Chemical ionization (CI-NH_3^+) was used to confirm the molecular weights. The mass spectra were confirmed by injection of standard compounds when available. Confidence in identifications is given in Table 1.

RESULTS

More than 100 compounds were detected in the polar fraction of the sunflower extract. The separation of all the compounds was obtained within 90 min. As an example, in Figure 1 are shown EAG recordings superimposed on the FID signal during a 13-min period of chromatography. The response to the standard stimuli was rather constant (mean = -1.7 mV, CI = -0.15 mV). The decrease of the EAG amplitude to the standard stimuli during the period of chromatography was small (less than 20%). Therefore, no correction was applied to the observed values of the EAGs.

From the 10 individuals for which complete recordings were obtained, a total number of 529 responses were observed during the chromatography: 95% of them were depolarizations (varying from -0.2 mV to -1.9 mV), and 5% were hyperpolarizations (varying from $+0.2$ mV to $+2$ mV). The responses to the different constituents were found throughout the chromatogram (Figure 2), except at the end when nonvolatile products were eluted (Figure 3). A larger number of responses occurred in the middle part of the chromatogram (retention times between 30 and 70 min) when compared to the remaining periods (Figure 2). Hyperpolarizations occurred throughout the period of analysis and were not related to particular peaks. The hyperpolarizations are evenly distributed along the chromatogram (χ^2, $P < 0.02$), and 85% of these hyperpolarizations occurred for only four individuals. The number of hyperpolarizations recorded from each bee could not be correlated with the sensitivity of each bee (mean value of the depolarizations recorded in each antenna) ($r^2 = 0.20$).

TABLE 1. IDENTIFICATION OF CONSTITUENTS OF SUNFLOWER POLAR FRACTION RELEASING EAG RESPONSES IN THE HONEY BEE.[a]

Peak number	Molecule		MW $CI-NH_3$[b]	Amount (% of extract)	Frequency of responses[c]	Mean EAG in mV + CI[d]
1	1-Pentene-3-ol	(a)		0.06	*	−0.5 + 0.4
2	3-Methyl-1-butanol	(a)	88	0.06	*	−0.9 + 0.4
3	*trans*-2-Hexenal	(a)	98	0.2	***	−0.6 + 0.2
4	1-Hexanol	(a)	102	0.07	**	−0.6 + 0.3
5	*cis*-Thujenol	(c)	152	0.01	*	−0.5 + 0.3
6	1,8-Cineole (eucalyptol)	(a)	154	0.5	*	−0.9 + 0.5
7	4-Thujanol (*trans*-sabinene hydrate)	(a)	154	1.2	*	−0.5 + 0.3
8	Myrtenal and myrtenol	(a)	150 152	0.8	**	−0.5 + 0.3
9	Unknown		166	0.08	**	−0.3 + 0.1
10	2,3,3-Trimethyl-epoxy cyclopentyl acetaldehyde	(c)	168	0.07	**	−0.4 + 0.2
11	Bornyl acetate	(a)	196	1.4	**	−0.5 + 0.3
12	Unknown		180	0.7	***	−0.5 + 0.3
13	Unknown		188	0.06	*	−0.4 + 0.3
14	β-Elemene	(a)	204	0.3	**	−0.6 + 0.6
15	Vanillin	(a)	152	0.6	**	−0.4 + 0.3
16	Branched C_{10} methyl ester		186	1.2	**	−0.5 + 0.3
17	Germacrene D	(a)	204	0.06	*	−0.4 + 0.2
18	Propiovanillone	(a)	186	1.4	**	−0.5 + 0.3
19	Caryophylene oxide	(a)	220	0.3	**	−0.8 + 0.2
20	Sesquiterpenic alcohol		220	3.9	**	−0.3 + 0.3
21	4-Hydroxy-2-methoxy cinnamaldehyde	(b)	178	1.2	***	−0.4 + 0.2
22	Unknown		266	0.07	**	−0.5 + 0.2
23	Tetradecanol	(d)		0.3	***	−0.5 + 0.2
24	Hexadecanoic acid	(a)	256	2.9	*	−0.3 + 0.3

[a] Relative intensities of the eight major ions of unidentified peaks: 9: 43(100), 41(72), 55(65), 83(44), 39(38), 67(36), 95(35), 99(34); 12: 41(100), 93(85), 39(84), 53(58), 105(53), 121(50), 91(46), 120(38); 13: 108(100), 41(54), 93(47), 95(37), 43(29), 39(27), 109(26), 55(23); 22: 123(100), 43(90), 41(12), 124(11), 55(6), 39(4), 77(4), 79(3).

[b] Letters in parentheses indicate the reliability of identification: (a) EI mass spectrum identical with literature spectra, MW confirmed by $CI-NH_3^+$ and identity confirmed by injection of standard compounds; (b) EI mass spectrum identical with literature spectra, MW confirmed by $CI-NH_3$; (c) EI mass spectrum in agreement with literature spectra based on eight major peaks, MW confirmed by $CI-NH_3$; (d) EI mass spectrum in agreement with literature spectra based on eight major peaks. The molecular weight is the result of the chemical ionization (reported when available).

[c] The frequency of responses is calculated from the number of antenna responding to the same stimulation: * = 80%, ** > 80%, *** = 100%.

[d] Mean calculated from depolarizations, CI is the confidence interval = standard error. $t = 0.05$).

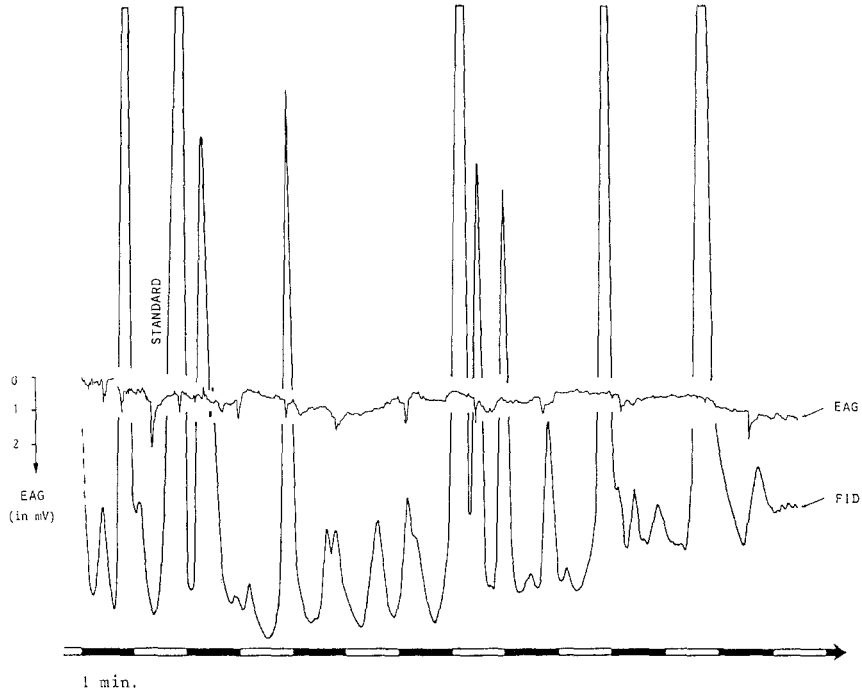

FIG. 1. Thirteen-minute sequence of the coupled signal synchronously recorded from the flame ionization detector (FID) and from a honeybee antenna (EAG). Standard is 1 ml of air odorized with hexanol 10:2 (v/v) in paraffin oil.

Twenty-four chemicals were found to stimulate the antennae for more than eight individuals. Amplitudes of the depolarizations recorded with these 24 compounds are plotted Figure 3. The total amount of these products account for 17% of the volatile constituents of the extract. The relative concentrations of these products range from 0.01% (thujenol cis) to 3.9% (sesquiterpenic alcohol, peak 20). Four compounds were commonly detected by all bees [*trans*-2-hexanal, peak 12 (unknown), peak 20 (sesquiterpenic alcohol), peak 22 (unknown); Figure 3, Table 1]. The variability of the amplitude of the recorded responses did not seem to be related to the retention time of the products: the higher variability observed (CI higher than 0.4 mV) occurred both at the beginning and the end of the experiment with 1-pentene-3-ol, 3-methyl-1-butanol, 1,8-cineole, and β-elemene.

The chemical identification of the 24 compounds found to be active in this experiment is given in Table 1. As expected, most of the stimulating compounds are oxygenated compounds belonging to different chemical classes. Fourteen of these identifications were confirmed by comparison with standard

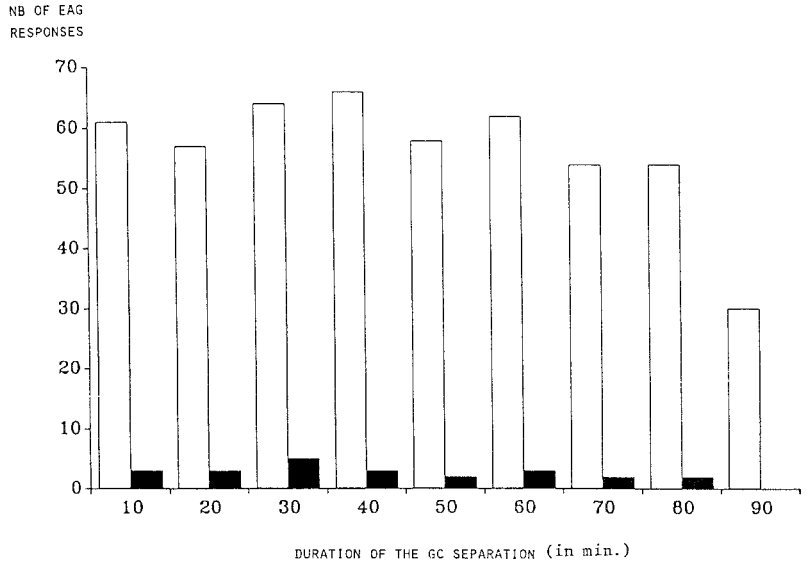

FIG. 2. Distribution of the EAG(s) recorded during the gas chromatographic separation of the polar fraction from a sunflower extract. White bars = depolarizations; black bars = hyperpolarizations.

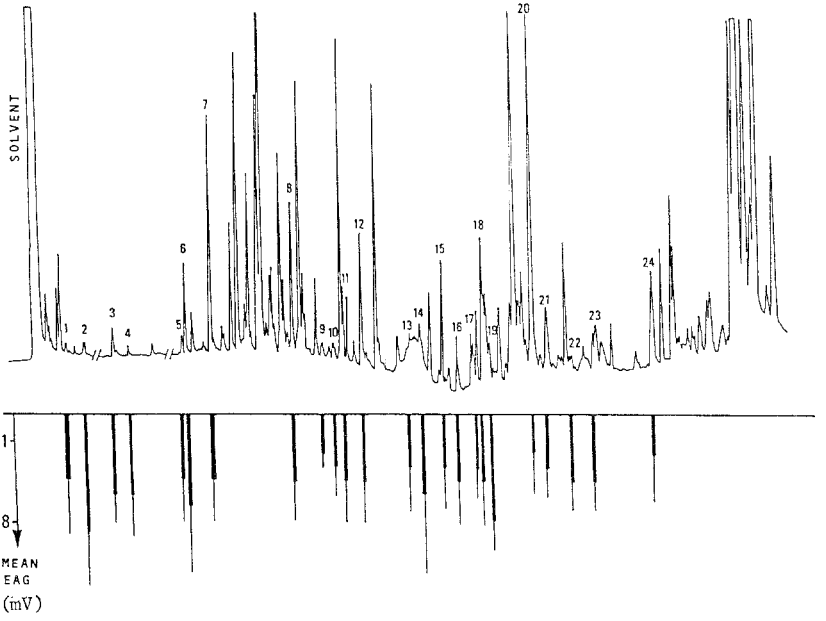

FIG. 3. Mean EAG responses of the honeybee antennae to compounds constitutive of a sunflower polar fraction. Numbers correspond to peak releasing responses in more than 80% of the recorded antennae. Thick bars represent the mean EAG, thin bars represent confidence intervals. Identification of chemicals is given in Table 1.

compounds; four of them were identified by comparison to published mass spectra; six compounds could not be identified precisely, although there is evidence that peaks 16 and 20 are a branched C_{10} methyl ester and a sesquiterpenic alcohol, respectively. The mass spectra of the four remaining products are listed in Table 1.

DISCUSSION

In a previous comparative study between the Soxhlet extraction technique and headspace sampling methods, Etiévant et al. (1984) stressed the validity of both methods for sunflower odor analysis. The Soxhlet extraction procedure has been preferred according to the higher concentration of chemicals collected as compared to air sampling. In the present work, more than 100 compounds have been separated from a polar fraction of sunflower solvent extract. From the same extract, Etiévant et al. (1984) separated a polar fraction of 47 compounds from a total of 84 products occurring; 31 polar compounds were identified. These differences may result from several reasons: our polar–apolar fractioning procedure could have been performed in slightly different conditions, degradation compounds may have appeared even if the initial extract was sealed in glass tube under nitrogen. The performance of our GC separation was higher, since we used a different bonded phase and an "on-column injector" that avoid thermal degradations and favors high-boiling compounds when compared to split–splitless injectors (Jennings, 1984; Jennings and Mehran, 1986).

In honeybees, detection of odors is thought to be achieved by olfactory receptor cells with broad overlapping quality spectra (Lacher, 1964; Vareschi, 1971). Since the EAG is believed to represent a summation of the receptor potentials of the responding cells (Kaissling, 1971), the exposure of antennal receptor cells to repeated stimuli may provoke an adaptation and a subsequent decrease of the EAG amplitude. Therefore the EAG activity needs to be interpreted with care. However, the slight decrease in response observed to the standard during the experiments suggests that the adaptation of receptors was limited, which may ensure the validity of such a screening.

In the present work, we used 8-days-old bees, which were grown in similar olfactory environment. Foraging bees (usually older than 8 days) may present differences in olfactory sensitivity because of age and also previous experience (Arnold and Masson, 1980; Masson and Arnold, 1984). Thus the spectrum of responses to the constituents of the sunflower odor might be different according to the age.

The EAGs recorded from the components of the polar fraction of the sunflower extract presented at a single concentration demonstrate that as many as 24 products were detected by the antennal receptor cells. The first four com-

pounds that release significant responses are only minor constituents of the extract (ranging from 0.06% to 0.2% of the extract). These are a short-chain aldehydes and alcohols (1-penten-3-ol, 3-methyl-1-butanol, 2-hexenal (*trans*), and 1-hexanol), which are common plant compounds also found in alfalfa flowers (Buttery et al., 1982), red clover flowers (Buttery et al., 1984), and ophrys flowers (Borg-Karlason and Tengo, 1986). Two of these compounds (3-methyl-1-butanol and 1-hexanol) are present in the alarm pheromone of honeybees and are highly effective in releasing alarm behavior (Collins and Blum, 1983). Tetradecanol is also mentioned as a volatile component of orchids flowers (Bergström, 1978). Ten of the 24 compounds detected by bees are terpenes and sesquiterpenes. Volatile terpenic compounds seem to occur frequently in flower aromas of the compositae (Etiévant et al., 1984; Flath et al., 1985; Buttery et al., 1986). These chemicals may be partly involved in flower recognition by bees (Waller et al., 1974; Pham Delègue et al., 1986). Caryophyllene oxide is probably a degradation product that may have appeared in the stored extract. It is nevertheless striking that bees are particularly sensitive to this product. This compound, identified in cotton plant extracts (Hedin, 1976), was also reported as releasing weak EAG responses in the boll weevil (Dickens, 1984). Some high-boiling-point compounds (e.g., linoleic acid) also elicited EAG responses in the bees. Their occurrence as olfactory stimulants may be due to the heating of these compounds in the gas chromatograph. Since they have very low volatility in natural conditions, it is doubtful whether they are important in olfactory communication and plant odor recognition.

The results presented here show a pattern of compound detection different from that found using foraging behavior experiments (Pham-Delègue et al., 1986). The 24 chemicals that elicited reproducible responses in our experiment are probably good candidates to be involved in the recognition of sunflower aroma by bees. Of these 24 compounds, more attention should be paid to the six compounds identified in the behavioral experiments mentioned above; namely, bornyl acetate, vanillin, propiovanillone, the products corresponding to peaks 9 and 10, and the branched methyl ester (peak 16), previously misidentified as methyl caprate.

In conclusion, coupling GC and EAG recordings allowed for rapid screening for components of the complex volatile mixtures in order to determine which of them might be used in olfactory recognition. However, the present results should be followed by behavioral tests to stress the role of the active components in blends. This approach can be useful for the tentative identification of a reduced chemical blend used by the bees for complex odor recognition. A better understanding of the role played by some component chemicals of the sunflower odor in the olfactory process could lead to their use in plant breeding programs in order to enhance the attractiveness of the plant and thus ensure pollination.

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ODOR VOLATILES ASSOCIATED WITH MITE-INFESTED BIN-STORED WHEAT

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Abstract—Tridecane, not previously reported in infested bin-stored grain, was associated with three mite species, *Acarus siro* (L.), *Aeroglyphus robustus* Banks, and *Lepidoglyphus destructor* (Schrank), which were introduced into 15.2% moisture content wheat stored in two unheated experimental bins in Manitoba during 1986–1987. The mites and volatiles were sampled at weekly to bimonthly intervals throughout the year. The mites produced tridecane all year, although they overwintered at low numbers. Ventilation of one bin at an airflow rate of 1 liter/sec/m^3 did not appear to affect mite survival and volatile production. Citral, an alarm pheromone for mites, was detected in mite-infested bin-stored grain. In laboratory studies all three mite species produced tridecane in wheat incubated at 20°C and 70% relative humidity for two weeks. *Acarus siro* and *Aeroglyphus robustus* reared at 25°C and 90% relative humidity for four weeks on wheat and fungus-inoculated wheat also produced tridecane. Production of tridecane by *A. robustus* and *L. destructor* is being reported for the first time.

Key Words—Odor volatiles, mites, stored wheat, *Acarus siro, Aeroglyphus robustus, Lepidoglyphus destructor*, Acarina, fungi, ventilation, tridecane, citral.

INTRODUCTION

Stored grain can be subjected to the deteriorative effects of various biotic agents including microflora, insects, mites, and rodents (Sinha, 1979). Mite-infested grain can be downgraded due to odor and taint, resulting in financial losses to

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producers and elevator companies. Prevention of such degradation is especially important to grain-exporting countries such as Canada and the United States if they wish to maintain their export markets and reputation as producers of high-quality grain.

Because of their minute size, compared to stored-grain insects, the presence of mite pests in a grain mass often remains undetected. Consequently, control measures are rarely applied until unacceptably high levels of infestation occur. Thus any chemical that is specifically produced only by mites and that could be detectable during early stages of mite development may provide an indication of infestation before serious damage to stored grain occurs.

A mite-specific volatile, tridecane, has been detected in large quantities from the headspace of laboratory cultures of stored-product mites (Curtis et al., 1981; Baker and Krantz, 1984). The collection method involved the adsorption of volatiles on a porous polymer over several days using large mite populations. This time-consuming method makes detection of tridecane in mite-infested grain difficult, particularly when levels of infestation are low. Another volatile, the alarm pheromone citral, was also detected in disturbed cultures of several stored-product mites (Baker and Krantz, 1984; Kuwahara et al., 1980). Neither of these volatiles was ever reported in unheated bins with mite-infested grain on a seasonal basis.

Ventilation of stored cereals with near-ambient air prevents spoilage of grain through drying and cooling and has become a common practice in Canada and elsewhere (Fraser and Muir, 1981; Metzger and Muir, 1983). Ventilation, however, can increase the number of some mite species in stored grain (Muir et al., 1977). The purging effect of airflow on the detection of mite volatiles in ventilated grain has not been studied.

The objective of this study was to monitor quantitatively odor volatiles characteristically produced by mite populations in nonventilated and ventilated grain bins containing mite-infested wheat and to determine in the laboratory whether there is a cause–effect relationship between mite species and the main mite volatile, tridecane.

METHODS AND MATERIALS

Stored-Grain Ecosystem: Bins, Wheat, Ventilation

The experimental bins, constructed inside an unheated machine shed, consisted of internally wax-coated cardboard cylinders 3.66 m high and 0.61 m diameter. The bins, sampling ports, and ventilation plenum have been previously described (Sinha et al., 1988).

Locally harvested Canada western hard red spring wheat [*Triticum aestivum* L., cv. Katepwa, 1985 crop, 20 and 25% moisture content (mc)] had been

ventilated and dried in 1986. The wheat was at 15.2% mc when placed in the experimental bins on July 21, 1986. The bin tops were covered with fine mesh and sealed with silicone to prevent escape of mites and infestation from external sources. Ventilation of grain with ambient air started on August 26, 1986, at a rate of 1 liter/sec/m^3 in one bin and continued for 16 weeks until December 18, 1986. Ventilation was resumed on June 12, 1987, for another six weeks until the end of experiment on July 31, 1987. The control bin was not ventilated.

Equal volumes of food media containing three species of stored-product mite pests, *Acarus siro* (L.) (Acarina; Acaridae), *Aeroglyphus robustus* Banks (Acarina; Glycyphagidae), and *Lepidoglyphus destructor* (Schrank) (Acarina, Glycyphagidae) (Hughes, 1976) were introduced into the grain in the control and ventilated bins. Mites were reared on yeast–wheat germ media (3:1, v/v) at 25 ± 1°C and 75 ± 2% relative humidity as part of the stock cultures maintained by the Agriculture Canada Research Station, Winnipeg. The estimated numbers of live mites introduced per bin location were: 443,000 for *A. siro*, 30,900 for *A. robustus*, and 97,350 for *L. destructor*. Mites were placed 15 cm below the grain surface at two points mid-way between the sides and center of the bin.

Samples for all analyses were taken through the bin port closest to the grain surface, using a metal probe. Samples for estimation of mite numbers were taken weekly, the frequency of sampling decreased to biweekly in November and to monthly in January 1987. Samples for volatiles analysis were taken monthly until November and bimonthly thereafter.

Mite Count. Mobile stages of adult and nymphal mites from grain samples were collected using Berlese funnels filled with 350 ml of grain and placed under 30-W lamps for 17 hr (Sinha, 1964). Heat from the lamps drove mobile mites from the grain into jars filled with 70% ethanol. A stereomicroscope was used to count the numbers of mites per sample and reported as mite number per 500 g of grain.

Grain Moisture Content and Temperature Measurement. Moisture content was determined on a wet-mass basis by oven-drying duplicate samples (15 ± 1 g) from each bin (American Society of Agricultural Engineers, 1983). Temperature was monitored using a Hewlett-Packard 3497A data acquisition unit (Hewlett-Packard, Avondale, Pennsylvania) and copper–constantan thermocouples located along the centerline of the bin.

Seed Germination and Microfloral Assessment. The rate of seed germination and microfloral infection were determined by filter-paper method using seeds surface-sterilized with 0.6% aqueous sodium hypochlorite solution (Wallace and Sinha, 1962; Mills et al., 1978). Plates were incubated in plastic bags at 22 ± 1°C for seven days. To break seed dormancy, seeds plated for germination were kept at 10°C for seven days before incubation. Infection levels were calculated as percentages of seeds on which microflora grew.

Determination and Analysis of Odor Volatiles

Samples of 500 g of wheat were flushed for 17 hr with N_2 at 60 ml/min flow and volatiles were preconcentrated on Chromosorb 105 adsorbent (Sinha et al., 1988). Volatiles were then desorbed into 100 μl acetone, and 1-μl aliquots were injected onto a Carbowax 20 M fused silica capillary column (50 m $\times$ 0.32 mm) in the splitless mode. The injector and flame ionization detector in the gas chromatograph were maintained at 200 and 250°C, respectively. Helium was used as carrier gas at a flow velocity of 45 cm/sec. Oven temperature remained at 37°C for 0.5 min, then increased to 77°C at a rate of 20°C/min, was held at this temperature for 5 min, and then increased to 150°C at a rate of 2°C/min.

Acetone solution of known volatiles (40 ng/μl) was injected daily and retention times were used for identification of peaks in new samples. Identities of peaks in samples were confirmed by cochromatography with known chemicals. The unknown volatiles were identified using electron-impact ionization and confirmed using methane positive-ion chemical ionization on a Hewlett-Packard 5985B quadruple gas chromatography-mass spectrometry system.

Diacetone alcohol (4-hydroxy-4-methyl-2-pentanone), present in the acetone used as a solvent, served as a convenient internal standard for quantifying the results. The peak area of each volatile was compared with the peak area of the diacetone alcohol for relative percent calculations. The mean relative percent of the volatile was calculated from two or three chromatographic runs.

Laboratory Experiments

Mite Volatiles on Wheat. Number 1 hard red spring wheat (*Triticum aestivum* L., cv. Katepwa, 1988 crop, 12% mc) was moisturized to 14% mc at ambient temperature and two weeks later stored at −10°C until used. A total of 400 adult mites were placed in round-bottom 0.5-liter flasks filled with 200 g of wheat. The flasks were closed with a cotton plug, covered with filter paper, then sealed with parafilm and incubated at 20 $\pm$ 1°C and 70 $\pm$ 2% relative humidity for two weeks. Two flasks per species and two control flasks containing only wheat were analyzed for mite volatiles in one to three chromatographic runs. These analyses were repeated, thus totaling four replicates for each treatment; standard errors were calculated from a maximum of 12 measurements.

Mite Volatiles on Fungus-Inoculated Wheat. Number 1 hard red spring wheat (*Triticum aestivum* L., cv. Katepwa, 1986 crop, 15% mc) was moisturized to 20% by adding sterile water in a mixer for 30 min and equilibrated for 24 hr at 10°C. Round-bottom 1-liter flasks were filled with 500 g of moisturized wheat, plugged with nonabsorbant cotton, and covered with aluminum foil. All flasks with moisturized wheat were autoclaved immediately at 121°C for 20 min, then stored at −15°C until required.

Fungal species *Alternaria alternata* (Fr.) Keissler, *Arthrobotrys* spp., *Eurotium herbariorum* (Wiggers)Link:Fr. (the perfect state of *Aspergillus repens*), *A. flavus* Link ex Fries, *A. versicolor* (Vuill.) Tiraboschi, *Penicillium cyclopium* Westling, *P. chrysogenum* Thom, *Fusarium moniliforme* Sheldon, and *Fusarium semitectum* (Cook) Sacc. were isolated on synthetic media from grain in a previous study (Tuma, 1988). A 1-ml suspension containing 10^6 spores (cells) was added into 1-liter flasks containing moisturized wheat. Duplicates of each treatment were incubated at 25°C and 90% relative humidity for one week, after which they were analyzed for microfloral volatiles (Tuma, 1988) and reused for mite incubation.

Mite Rearing. Large populations of two mite species, *Acarus siro* (L.) and *Aeroglyphus robustus* Banks were reared on a yeast-wheat germ media (3:1, v/v) at 20 ± 1°C and 70 ± 2% relative humidity for six weeks. A lot of 1000 adult mites was counted and placed, using a sable-hair brush, into each flask containing fungus-inoculated wheat. Flasks were incubated at 25 ± 1°C and 90 ± 2% relative humidity for four weeks. The standard error values presented in Table 1 were calculated from a maximum of six chromatographic measurements of volatiles collected from two flasks with 500 g of wheat.

TABLE 1. MEAN TRIDECANE COLLECTED FROM HEADSPACE OF TWO FLASKS CONTAINING MITE-INFESTED WHEAT WITH OR WITHOUT FUNGAL INOCULATION AFTER INCUBATION AT 25°C AND 90% RELATIVE HUMIDITY FOR 4 WEEKS

Mite species	Fungal species	Tridecane (%) (X ± SE)
Acarus siro (noninoculated)		404.7 ± 35.2
Acarus siro 7-day wheat (OA)[a]		476.8 ± 63.1
Acarus siro	*Aspergillus repens*	380.5 ± 15.1
Acarus siro	*Aspergillus repens* (OA)	423.9 ± 25.3
Aeroglyphus robustus	*Aspergillus repens*	56.9 ± 5.5
Acarus siro	*Penicillium cyclopium*	n.d.[b]
Acarus siro	*Penicillium cyclopium* (OA)	n.d.
Aeroglyphus robustus	*Penicillium cyclopium*	8.4 ± 1.4[c]
Acarus siro	*Penicillium chrysogenum* (OA)	n.d.
Acarus siro	*Aspergillus versicolor* (OA)	n.d.
Acarus siro	*Aspergillus flavus* (OA)	n.d.
Acarus siro	*Fusarium moniliforme* (OA)	334.9 ± 4.3
Acarus siro	*Fusarium semitectum* (OA)	1030.5 ± 5.9
Acarus siro	*Arthrobotrys* spp. (OA)	221.2 ± 11.2
Acarus siro	*Alternaria alternata* (OA)	489.9 ± 88.0

[a] OA, previously analyzed for odor volatiles
[b] n.d., not detected
[c] Tridecane detected and calculated from one flask only.

One-Way Analysis of Variance. The data from the laboratory study on mite volatiles in fungus-inoculated wheat were statistically analyzed with one-way analysis of variance (ANOVA) using Scheffe's and Student-Newman-Keuls' (SNK) multiple comparison methods (Statistical Analysis System, 1985).

RESULTS

Bin-Stored Wheat Ecosystem

Physical Conditions. The initial grain temperature of 26°C decreased to 18°C by the end of August. Grain temperatures in the ventilated bin were usually a few degrees lower than those in the nonventilated control bin, presumably because of the movement of cold outdoor air. Temperatures in both bins generally followed the decreasing ambient temperature to 12–14°C in September, 6–8°C in October, and −10°C in November, at which level the grain temperature remained unchanged during the winter months. Grain temperatures then increased with ambient temperature to 3°C in March 1987, 11°C in April, 16°C in May, 22–25°C in June, and finally to 26°C when the experiment was terminated on July 31, 1987. Grain moisture content in control and ventilated bins fluctuated between 14.1 and 15.5% during the storage period.

Mite Populations and Odor Volatiles. Three mite-specific volatiles were detected in both nonventilated control and ventilated bin-stored wheat ecosystems. These were tridecane, neral, and geranial; the last two volatiles are components of the pheromone citral and occurred in lower quantities than tridecane. Occurrence of three mite species *Acarus siro, Aeroglyphus robustus*, and *Lepidoglyphus destructor* coincided with the incidence of tridecane, which was detected during the entire storage period. Although the number of *Acarus siro* introduced was 14 times higher than that of *Aeroglyphus robustus*, the ratio of population sizes between the two species reversed as time proceeded. During the winter months when grain temperature reached −10°C, *Aeroglyphus robustus* had the highest survival of the three introduced mite species.

Acarus siro was the most abundant mite species in the control bin during August–October 1986 (Figure 1A). This mite was not detected during the winter months, and its numbers remained relatively low during the rest of the experiment. Two other introduced mite species, *Lepidoglyphus destructor* and *Aeroglyphus robustus* fluctuated within their original population levels during August–October. In November 1986 the numbers of *Aeroglyphus robustus* (560 per sample) far exceeded those of the other two mite species. *A. robustus* remained as the only active species during the winter. In the spring and summer, *A. robustus* was the most common and abundant mite species, increasing sharply in numbers once in March and then again in April 1987; it had the highest survival rate.

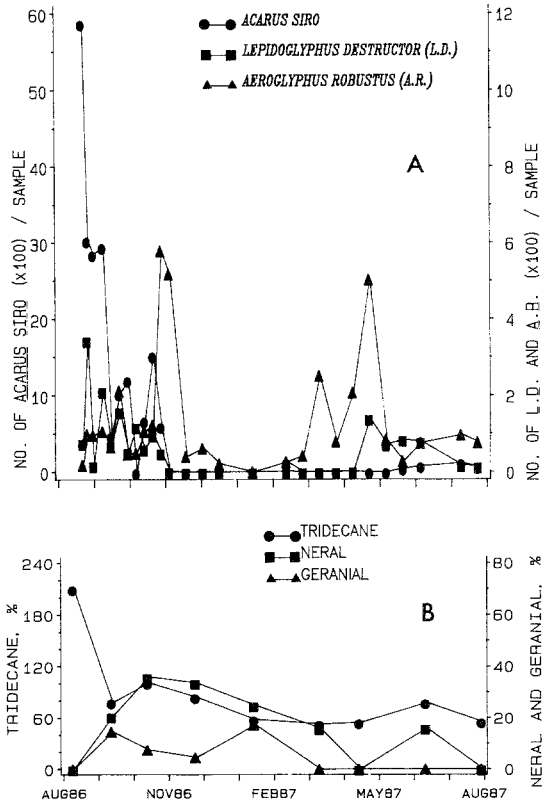

FIG. 1. Seasonal fluctuations of mite populations and mite volatile levels in 500-g samples from nonventilated control bin containing 15.2% mc wheat in Winnipeg, Manitoba, during 1986–1987. (A) Three species of stored-grain mites; (B) mite volatiles expressed as relative percent of diacetone alcohol internal standard.

Tridecane, initially recorded at 210% in August 1986, occurred within a 50–110% range during September 1986–July 1987 in the control bin (Figure 1B). Neral and geranial were detected during September 1986–January 1987. Only neral was detected twice at the 15% level during March–July 1987.

In the ventilated bin, *Acarus siro* was the most abundant mite species during August–October 1986 (Figure 2A). This mite species was not detected during the winter and spring, but it reappeared at low levels in the summer. In November 1986 the numbers of *Aeroglyphus robustus* (1080 per sample) far exceeded those of the other two species; it remained as the only active species during winter. *L. destructor* increased to its highest level in April 1987. Of the three introduced mite species, *A. robustus* had again the highest survival rate.

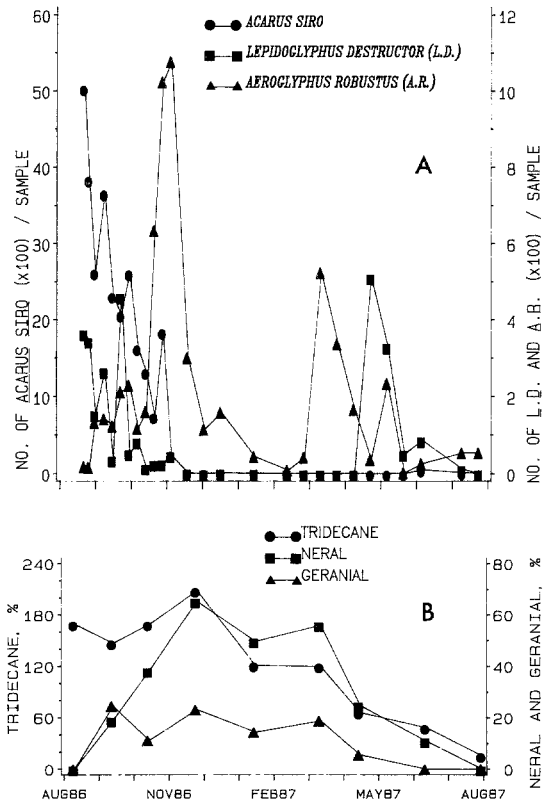

FIG. 2. Seasonal fluctuations of mite populations and mite volatile levels in 500-g samples from ventilated bin containing 15.2% mc wheat in Winnipeg, Manitoba, during 1986–1987. (A) Three species of stored-grain mites; (B) mite volatiles expressed as relative percent of diacetone alcohol internal standard.

In the ventilated bin, tridecane increased to 210% in November 1986; henceforth, the level declined steadily to 20% in summer (Figure 2B). Neral and geranial also peaked in November and then decreased to undetectable levels in July 1987.

Seed Germination and Microflora. There were no substantial differences in seed germination and internal infections between control and ventilated bins. Seed germination fluctuated between 48 and 88%. The level of infection by storage fungi was between 48 and 84% for *Aspergillus glaucus* gr. and between 20 and 56% for *Penicillium* spp. The level of infection by a field fungus *Alternaria alternata* was between 0 and 32% and that by bacteria between 8 and 72%.

Laboratory Experiments on Mite Odor Volatiles

Mite Volatiles on Wheat. Tridecane was detected at 14.6–29.1% in all three mite species after two weeks of incubation at 20°C and 70% relative humidity. The mean percent of tridecane level with standard errors for *Acarus siro* was 20.6 ± 1.8, for *Lepidoglyphus destructor* 14.6 ± 2.0 and for *Aeroglyphus robustus* 29.1 ± 4.1.

Mite Volatiles on Fungus-Inoculated Wheat. Tridecane was not detected in flasks containing fungi-inoculated wheat (Table 1) (Tuma, 1988) except in one containing *A. repens*-inoculated wheat; this flask, showing 78.4% tridecane, was infested by a contaminant stored-product mite, *Tyrophagus putrescentiae* (Schrank). When *Acarus siro* was introduced separately into flasks with noninoculated wheat, the mites contaminated the wheat with *Aspergillus repens* and sporadically with *Penicillium* spp.

In wheat with *Aspergillus repens* and *Acarus siro*, the tridecane level (380.5%) was seven times as high as that in wheat with *A. repens* and *Aeroglyphus robustus* (56.9%). In wheat inoculated with *Aspergillus repens, Aeroglyphus robustus* had a lower reproduction than *Acarus siro*. In wheat inoculated with *Penicillium* spp. and *Acarus siro* or *Aeroglyphus robustus*, no tridecane occurred except in one flask, which had an 8.4% tridecane level; the mite populations in all these flasks were declining and in poor condition. Condensation in the flasks containing wheat inoculated with *P. cyclopium* and *P. chrysogenum* could have negatively affected the survival of both mite species.

In wheat with *Fusarium semitectum* and *Acarus siro*, tridecane occurred at a 1030.5% level; this level was significantly different (ANOVA, $P < 0.05$) from those in all other treatments. Fungal spores were eaten by mites in all flasks in which high levels of tridecane were recorded. Based on tridecane levels, fungal substrates that provide the best dietary source for *Acarus siro* include: *Fusarium semitectum, Alternaria alternata, Aspergillus repens, Fusarium moniliforme*, and *Arthrobotrys* spp. Another mite-specific volatile, perillen, was detected in most flasks that produced tridecane. The perillen levels, ranging from 23 to 191%, were always lower than those of tridecane in all flasks.

DISCUSSION

Tridecane was detected in bin-stored wheat only when it was infested with the stored-product mites, *Acarus siro, Aeroglyphus robustus*, and *Lepidoglyphus destructor*. The laboratory study demonstrated that tridecane could be produced by all three mite species. The production of citral components and perillen by mites was always less frequent and in lower quantities than the production of tridecane.

The highest levels of tridecane occurred during the first four months of the

12-month storage period when the mite counts from grain samples were also high. *Acarus siro*, which was most abundant during the first three months of storage, was replaced by *Aeroglyphus robustus* during subsequent months. The high survival rate of the active stages of *Aeroglyphus robustus* could have accounted for most of the tridecane detected during winter. Because both *Acarus siro* and *L. destructor* overwinter in the cold-tolerant nonfeeding hypopus stage; mites in this stage are inadequately extracted by the Berlese method used in this study (Sinha, 1964). Thus, the numerical dominance of *Aeroglyphus robustus* over the two other mite species could be misleading. Neral and geranial, detected in mite-infested grain, probably resulted from disturbing the mites during handling in the laboratory, as mites do not yield large quantities of these volatiles unless alarmed (Baker and Krantz, 1984; Kuwahara et al., 1980).

Laboratory-incubated *Acarus siro* produced tridecane at a 20.6% level after two weeks and at 404.7 and 476.8% levels after four weeks. This substantial difference could have resulted, at least in part, from differences in incubation time, moisture content of wheat, and initial population size. Contamination of media with *Aspergillus repens* could be another factor responsible for higher tridecane levels in 19% mc wheat, after a four-week incubation. This wheat was more susceptible to fungal contamination than a similar but contaminant-free 14% mc wheat, after a two-week incubation. This contamination could have provided an additional dietary source for the mites, thereby enhancing reproduction and subsequent tridecane production.

Both laboratory-incubated mite species, *Acarus siro* and *Aeroglyphus robustus*, reared on wheat and wheat inoculated with fungal species produced tridecane. Curtis et al. (1981) also observed this phenomenon for *Acarus siro*. The production of tridecane by *Aeroglyphus robustus*, however, has not been previously reported. *Aeroglyphus robustus* is a well established mite pest of stored grain in Canada (Sinha, 1966) and a native of North America (Banks, 1906); its volatile production has not been previously studied. Tridecane was also produced in *Tyrophagus putrescentiae*-contaminated wheat, but it was not detected in bin-stored wheat naturally infested with *Tarsonemus granarius* Lindquist. This natural infestation occurred in all bins, but the numbers were not reported because it was presumed that the presence of *T. granarius* did not affect the tridecane production. Further research is needed to assess the tridecane production capabilities of other species of stored-grain mites reared under laboratory conditions.

Abundance of tridecane produced by mites differed substantially when mites were reared on wheat infected with fungi. The ranking of fungal diets for *Acarus siro*, according to tridecane production levels, was: *Fusarium semitectum, F. moniliforme, Aspergillus repens*, and *Arthrobotrys* spp. No neral or geranial was detected from mites in our laboratory experiments; absence of

these volatiles could have resulted from not disturbing the flask contents and mites prior to analysis (Baker and Krantz, 1984; Kuwahara et al., 1980). In our studies lesser quantities of perillen than tridecane were detected in the headspace of flasks containing media with *Acarus siro*; this finding is in agreement with that of Curtis et al. (1981).

Little is known about the biological effects of these volatiles on various species of fungi and mites. There is some evidence, however, that citral and citral-containing mite extracts inhibit fungal proliferation (Cole and Blum, 1975; Matsumoto et al., 1979) and function as alarm pheromones against mites (Kuwahara et al., 1980).

Further studies on the specificity and temperature dependency of tridecane are necessary before tridecane can be tested as a possible bioindicator of early infestations in stored grain. Stored grain is a dynamic ecosystem and must be monitored in a sophisticated manner if sound strategies to prevent degradation of grain quality are to be formulated.

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ANTIBIOTIC PROPERTIES OF PORCUPINE QUILLS

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Abstract—Porcupine quills possess antibiotic properties. The antibiotic activity is associated with free fatty acids (but not neutral lipids) coating the quills. Extracts of quill fatty acids strongly inhibited the growth of six gram-positive bacterial strains. No growth inhibition was observed against four gram-negative strains. Free fatty acids made up 18.6% of total quill lipids in samples collected in the summer, and 5.5% of total lipid in samples collected in the winter. The fatty acids were separated and identified (as the methyl esters) by gas–liquid chromatography and mass spectroscopy. Major components of a complex mixture included 14-methylpentadecanoic, 9-hexadecenoic, hexadecanoic, and 9-octadecenoic acids. It is suggested that porcupines benefit from the quill fatty acids: evidence from healed fractures of major skeletal components (35.1% incidence in 37 skeletons examined) suggests that porcupines fall relatively frequently from trees. Quill antibiotics may limit self-injury suffered in such falls.

Key Words—*Erethizon dorsatum*, porcupine, fatty acids, antibiotics, healed fractures.

INTRODUCTION

Wounds produced by quills of the North American porcupine (*Erethizon dorsatum*) rarely become infected (Shadle, 1955; Maser et al., 1981; Strickland et al., 1982; Maser and Rohweder, 1983; Roze, 1985). In contrast, spine penetration from other spine-defended animals such as echinothurid and diademid sea urchins (Echinodermata: Echinoidea) frequently produces secondary infections (Earle, 1940, 1941). Because porcupine quill wounds typically remain uninfected, a quill antibiotic factor was suspected. Porcupine quills are covered

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with a greasy coating; the search for an antibiotic factor focused on this lipid material.

Quilled or spine-covered animals would be expected to evolve defenses that inflict maximal damage to predators. To understand the apparent limitation placed on quill defense in the porcupine, the hypothesis was examined that the animals may suffer self-quilling as a result of accidents while foraging in the small outer branches of food trees.

METHODS AND MATERIALS

Lipid Composition. Porcupines were live-trapped in a hardwood–hemlock (*Tsuga canadensis*) forest in Greene County, New York, and anesthetized with ketamine HCl. Approximately 50–100 quills, weighing ca. 1.5 g, were removed with forceps from the lower backs and tails of each animal and stored at −20°C until used. Quills of individual animals were extracted with *n*-hexane (10 ml/g quills) at room temperature, and the extract clarified by centrifugation. The hexane extract was evaporated to dryness under a stream of nitrogen, weighed, redissolved in hexane, and separated into a free fatty acid fraction and a neutral lipid fraction.

The free fatty acid fraction was obtained by shaking 15 ml of hexane extract with 13 ml of 0.2 N aqueous KOH. The organic phase contained neutral lipids. The aqueous phase was acidified with 0.3 ml conc. HCl and extracted with 10 ml *n*-hexane. The hexane reextract containing free fatty acids was removed and concentrated under a stream of N_2. For gas chromatographic analysis, heptadecanoic acid (Sigma Chemical Co.) was added as an internal standard, and the fatty acids methylated with BF_3/MeOH (Supelco methylation kit 3-3020).

The esterified fatty acid content of the neutral lipid fraction was measured by transesterifying 1 ml of neutral lipid fraction with 1 ml 0.2 N (*m*-trifluoromethylphenyl) trimethylammonium hydroxide in MeOH (Meth-Prep II, Alltech 18007) plus 1 ml CH_3Cl, and treating for 20–30 min at room temperature. Trimyristin (Sigma Chemical Co.) was used as the internal standard. Under these conditions, esters of fatty acids were transesterified to fatty acid methyl esters (FAMEs). Complete separation of the free fatty acid and neutral lipid fractions was indicated by the absence of myristyl FAME in free fatty acid fraction, and C-17 FAME in neutral lipid fraction.

FAME analyses were carried out with a Hewlett-Packard 5988A gas chromatograph–quadrupole mass spectrometer (GC-MS) with a Hewlett-Packard 1000 data system. A 30-m × 0.25-mm-ID fused silica capillary column with a DB-5 bonded methyl silicone stationary phase (J&W Scientific) was used with helium carrier gas and splitless injection. Initial column temperature was held

at 35°C for 1 min, then programmed at 20°C/min to 150°C, where it was held for 2 min, and then programmed at 2.5°C/min to 225°C, with a final hold of 15 min. GC-MS results were confirmed using a 50-m × 0.32-mm Hewlett-Packard cross-linked Carbowax 20 M column. Compound identification was based on cochromatography of sample components with standards, comparison of mass spectra of sample components with those of standards, or the NBS-Wiley spectral library (installed in the Hewlett-Packard 1000 system), and mass spectral interpretation based on Odham and Stenhagen (1972) and Ryhage and Stenhagen (1960). FAME standards were obtained from Supelco (4-7028), Sigma (189-1, 189-4, 189-6, and C-17), and Regis Chemical (Qualmixes BR2 and BR4).

The triglyceride content of the neutral lipid fraction was measured by coupled enzymatic assay of glycerol generated by saponification of the neutral lipids by KOH–EtOH (Sigma triglyceride kit 320-A). In addition, the neutral lipid fraction was separated qualitatively by thin-layer chromatography (TLC) on silica gel G, using three solvent systems (1, hexane–ethyl ether–HAc 70 : 30 : 1; 2, toluene; 3, $CHCl_3$–EtOH 100 : 1).

Antimicrobial Effects. Bacteriostatic properties of the neutral lipid and fatty acid fractions were tested at a concentration of 50 μg/ml, after removal of hexane under a stream of N_2. Nonclostridial bacterial cultures were grown aerobically in nutrient broth at 37°C in a rotary water-bath shaker. Growth was followed for 4–6 hr by absorption at 600 nm, as measured by a Bausch and Lomb Spectronic 20 spectrophotometer. Clostridial species (American Type Culture Collection) were grown in capped vials containing 0.05% thioglycollate in fluid broth (Difco Laboratories). Growth was followed for 48 hr and measured as above.

Healed Fractures. To estimate the incidence of serious injuries in porcupines, 37 available skeletons were examined for evidence of healed fractures. Porcupine skeletons were examined from the collections of four museums (American Museum of Natural History, New York, New York = AMNH; Field Museum, Chicago, Illinois = FM; University of Wisconsin Zoological Museum, Madison, Wisconsin = UWZM; and U.S. National Museum of Natural History, Washington, DC = USNM) and the Queens College (QC) collection. Only complete, non-zoo skeletons were examined. Porcupines with deciduous premolars (Pls) were classified as subadults (SA), and those with permanent Pls as adults (A) (Earle and Kramm, 1980; Sutton, 1972).

Because rib and phalangeal injuries are difficult to classify (Bulstrode et al., 1986), they were excluded from the analysis. For comparison, 45 skeletons of the woodchuck (*Marmota monax*) and 39 skeletons of the raccoon (*Procyon lotor*) were examined. Results are presented as means ± SD. Statistical procedures follow Sokal and Rohlf (1981).

RESULTS

Lipid Composition. Hexane-extractable quill lipids averaged 8.0 ± 5.7% w/w of quill weight. Table 1 lists average concentrations of principal components (>2% w/w) present in the free and esterified fatty acid fractions of seven different animals. The amounts are normalized to 100% for comparison. Minor components (<2% each) included tridecanoic (13:0), 12-methyltridecanoic (iso-14:0), tetradecanoic (14:0), 14-methylhexadecanoic (iso-17:0), octadecanoic (18:0), and 16-methylheptadecanoic (iso-18:0) acid, as well as six unidentified components. The close similarity between the transmethylated and free fatty acid fractions suggests that the latter arise from hydrolysis of fatty acid esters. A straight-chain saturated fatty acid (16:0) appears to be released preferentially.

The free fatty acid content of quill grease varied inversely with esterified fatty acid content ($r = -0.915$, $P < 0.05$). Quill grease from animals sampled in August averaged 18.6 ± 5.1% free fatty acid; January–February samples averaged 5.5% ± 1.5% free fatty acid ($t = 4.98$, $P < 0.01$) (Figure 1). Triglyceride content was proportional to neutral lipid content and averaged 5.6 ± 0.4% w/w of neutral lipid in both summer and winter samples (expressed as triolein equivalent). TLC showed presence of triglycerides plus at least nine additional components in the neutral lipid fraction.

Bacteriostatic Properties of Quill Lipids. The neutral lipid fraction proved inactive against all microorganisms tested. The free fatty acid fraction, on the other hand, showed strong growth inhibition of six of 10 bacterial strains tested (Table 2). All sensitive strains were gram-positive. The minimal inhibitory concentration, measured against *S. aureus*, was 6.2 μg/ml.

Healed Fractures. Of 37 porcupine skeletons examined (28 adults, 9 subadults), 13 (35.1%) showed healed fractures of bones other than ribs or phalanges. Healed injuries included the following: tibia and fibula (QC); femur, scapula, and dislocated rib 6 (AMNH 120842); ischium (AMNH 128710);

TABLE 1. MAJOR FREE AND ESTERIFIED FATTY ACIDS (FAS) IN PORCUPINE QUILL LIPIDS

Compound	Free FAs (wt %)	Esterified FAs (wt %)
14-Methyl pentadecanoic acid (iso-16:0)	5.2 ± 2.4	6.3 ± 2.3
9-Hexadecenoic acid (16:1)	25.7 ± 4.2	27.2 ± 5.8
Hexadecanoic acid (16:0)	54.8 ± 10.2	43.9 ± 11.4
9-Octadecenoic acid (18:1)	14.3 ± 5.8	22.6 ± 6.3

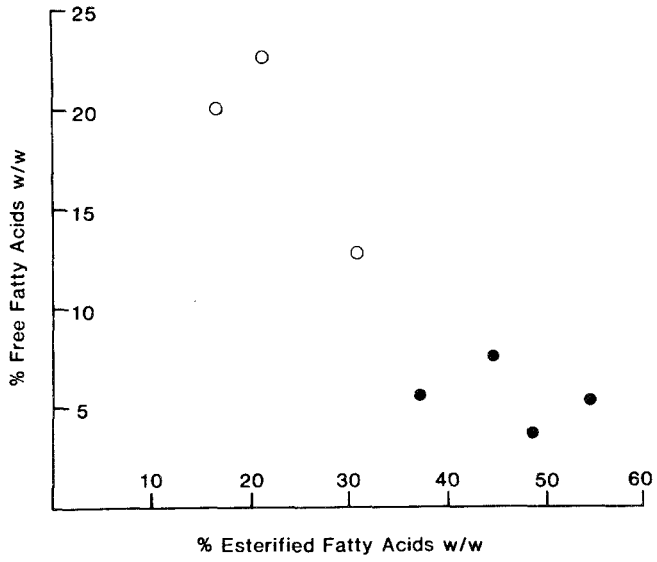

FIG. 1. Correlation between free and esterified fatty acid contents of quill grease. (○) Samples collected in August; (●) samples collected in January–February.

maxilla (AMNH 150093); humerus (AMNH 165044); radius (UWZM 16433); sacrum, femur, and acetabulum (UWZM 20181); femur and acetabulum (UWZM 21650); ilium (FM 20345); pubis, ischium, and skull (FM 47173); tibia (USNM 21515); femur and scapula (USNM 88619); and femur (USNM

TABLE 2. BACTERIAL GROWTH RESPONSES TO PORCUPINE QUILL FATTY ACIDS

Species	Growth inhibition	Gram stain
Bacillus cereus	+	+
B. subtilis	+	+
Clostridium butyricum	+	+
C. novyi	+	+
Staphylococcus aureus	+	+
Streptococcus faecalis	+	+
Enterobacter aerogenes	–	–
Escherischia coli	–	–
Proteus mirabilis	–	–
P. vulgaris	–	–

192546). All but one of the healed fractures (USNM 88619) were found in adult porcupines.

The following porcupine specimens were examined and found free of healed fractures: AMNH 63759, SA; AMNH 97646, A; AMNH 97647, SA; AMNH 121197, A; AMNH 126094, A; AMNH 146825, SA; AMNH 147530, SA; AMNH 185327, A; FM 20343, A; FM 43299, A; FM 58811, SA; FM 124114, A; UWZM 20223, A; UWZM 21657, A; UWZM 24049, A; USNM 676, A; USNM 822, A; USNM 21159, A; USNM 21516, A; USNM 88617, SA; USNM 90475, A; USNM 90477, SA; USNM 192541, A; USNM 249955, SA.

Of 45 woodchuck skeletons examined, three (6.7%) showed evidence of healed fractures: radius, accompanied by arthritic disease of same (UWZM 22705); ilium, tibia, and sacrum (USNM 20765); and tibia (USNM 514747). The following woodchuck skeletons were examined and found free of healed fractures: UWZM 4059; UWZM 13950; UWZM 18563; UWZM 20400; UWZM 20459; UWZM 20620; UWZM 22222; UWZM 22704; UWZM 22707; AMNH 35615; AMNH 38873; AMNH 67882; AMNH 70014; AMNH 97386; AMNH 98963; AMNH 126079; AMNH 179937; AMNH 180314; AMNH 207022; AMNH 235277; AMNH 235648; FM 41087; FM 53201; FM 121211; USNM 604; USNM 88618; USNM 191374; USNM 191376; USNM 258735; USNM 260385; USNM 291554; USNM 293895; USNM 347666; USNM 349705; USNM 349710; USNM 349711; USNM 396282; USNM 397378; USNM 397379; USNM 397400; USNM 506225; USNM 514748.

Of 39 raccoon skeletons examined, all from the AMNH collection, four (10.2%) showed healed fractures: femur, 208968; radius, 245491; scapula, 253188; tibia and fibula, AMNH unnumbered September 15, 1928. The following skeletons were examined and found free of fractures: 93145; 121198; 123880; 145143; 173897; 173898; 173899; 183363; 187080; 235185; 235189; 235209; 236135; 236137; 237438; 237439; 238270; 238271; 238272; 238273; 238274; 238275; 238276; 238278; 238458; 238565; 238603; 243100; 245493; 245494; 245495; 245498; 245629; 245645; 255651.

The incidence of healed fractures in porcupines is significantly higher than in woodchucks or raccoons ($\chi^2 = 11.66$, $df = 2$, $P < 0.005$).

DISCUSSION

The antibiotic activity of fatty acids has long been recognized (Glassman, 1948; Wyss et al., 1945). Fatty acids kill gram-positive bacteria and many fungi, but are not active against gram-negative bacteria (Wyss et al., 1945). The mode of action is thought to involve an attack on the bacterial cell membrane (Esplin, 1970). Presumably gram-negative microorganisms escape attack

because of their lipid-rich outer envelopes (Stanier et al., 1976). In humans, free fatty acids are released by enzymatic attack on triglycerides and wax esters by normal flora such as *Propionibacterium acnes* (Sherris, 1984a; Marples et al., 1970). On human skin, the bacterial lipases preferentially release straight-chain saturated fatty acids (Bore et al., 1982).

The higher free fatty acid content of summer quill grease samples is consistent with a temperature-dependent enzymatic hydrolysis release. Likewise, an enzymatic hydrolysis release mechanism is supported by the observed inverse relationship between free fatty acid and esterified fatty acid contents of quill grease (Figure 1).

The composition of porcupine quill lipids differs significantly from the typical mammalian pattern. Skin lipids of most mammals contain only minute amounts of free fatty acids or triglycerides. The notable exception is human skin, where fatty acids make up about 30% of total lipids (Lindholm et al., 1981; Nicolaides, 1974). In most mammals, major components of skin surface lipids include mono- and diester waxes, internal lactones, sterol esters, and free sterols. Suggested functions of skin surface fatty acids in humans include reduction of bacterial and fungal infections of the skin (Sauer, 1980; Sherris and Ray, 1984).

The greasy coating of porcupine quills may serve multiple functions such as keeping quills stiff and water-resistant and aiding in individual recognition. For the fatty acid component of the quill grease, we here suggest an additional function: limitation of self-injury following impalement on the animal's own quills. Evidence presented from healed fractures suggests that porcupines suffer injuries more often than nonarboreal (woodchuck) or arboreal (raccoon) animals of comparable body weight.

Others have also noted that porcupines may suffer injuries in the wild. Dodge (1967) found healed fractures in legs, hips, and ribs, as well as broken incisors, injured eyes and ears, hernias, and miscellaneous soft-tissue injuries. Most such injuries are consistent with falling from trees. Curtis and Kozicky (1944) observed a full-grown porcupine falling from a poplar tree. Marshall (1951) found a porcupine dead in a double-stemmed tamarack tree. The animal had apparently slipped while climbing and had wedged itself sideways, just behind the forelimbs, in the V of the trunks. It had been unable to extricate itself from this position.

During seven years of following radiotelemetered porcupines in the Catskills in New York, additional circumstantial evidence has suggested that porcupines can fall from trees: broken branches in feeding trees, quill masses under feeding trees, and external as well as internal injuries suggestive of falling (U. Roze, unpublished observations).

The incidence of healed fractures reported here for porcupines is comparable to figures reported for other arboreal and semiarboreal species. Schultz

(1956) found that in wild *Hylobates*, *Pongo*, *Pan*, and *Gorilla*, the frequency of adult specimens with at least one healed fracture was 33%, 34%, 18%, and 21%, respectively. The figures include phalangeal and rib fractures. Kano (1984) interprets the Schultz data as suggesting that degree of arboreality in apes is correlated with frequency of accidents.

Clarke and Glander (1984) offer more direct evidence for a correlation between arboreality and accidents, by the observation that Costa Rican howler monkeys (*Alouatta palliata*) can fall from trees, sometimes with fatal results. They speculate that the much higher infant mortality among howler males (59%) compared to females (11%) results from more vigorous play in trees and an earlier age of independence.

This suggests that accidents result not from arboreal behavior per se but from risk-taking behavior in trees. The lower incidence of healed fractures observed in raccoons may reflect the fact that raccoons use trees primarily for rest and denning, and therefore confine their use to the trunk and large branches (Lotze and Anderson, 1979; Stuewer, 1943; Urban, 1970).

During summer in a deciduous forest habitat, porcupines may be in trees almost continuously, using them for diurnal rest and nocturnal feeding in the thin outer leaf-bearing branches of the trees. Tree use declines in winter as animals spend the bulk of their time in dens, emerging only for nightly feeding bouts. These are focused on the inner bark of trunks and larger branches (Dodge, 1967; Roze, 1987). Hence the animals encounter higher risks in summer. Data presented in Figure 1 show that porcupine quills carry higher fatty acid concentrations during this time.

It is postulated that a significant fraction of porcupines who fall would impale themselves on their own quills, with consequent risk of infection. Self-quilling has been observed in many cases of attempted capture, because of the flailing of the animal (U. Roze, unpublished observations).

In such cases of self-impalement on quills, how effective an antibacterial defense might the quill fatty acids be expected to offer? Much of the quill damage would be self-limited, since porcupines are skilled in removing embedded foreign quills (Dodge, 1967). However, quill tips may break off and travel beyond reach through internal tissues (Shadle, 1947; Vincent and Owers, 1986). Under such conditions, the bacteria associated with the quill tip would be in position to start an infection.

In humans, *Staphylococcus aureus* (Table 2) is the most common pathogen associated with contaminated puncture wounds, although the major threats are tetanus and gas gangrene, caused by the gram-positive *Clostridium tetani* and *C. perfringens*, respectively (Isenberg and D'Amato, 1985; Sherris, 1984b). These organisms are associated with foreign bodies such as splinters, soil particles, and fecal material. The den environment of the porcupine typically contains accumulations of fecal material. Clostridia may multiply under the

anaerobic conditions of deep puncture wounds. Mixed infections, frequently derived from the normal flora, and consisting of an anaerobic gram-negative or gram-positive organism plus a facultative anaerobe such as the gram-positive *S. aureus*, may also represent a threat. Under puncture-wound conditions, growth of the anaerobes is favored because of local oxygen depletion by the aerobe (Sherris, 1984b; Gorbach, 1974). Quill fatty acids, by inhibiting the gram-positive members of such an association, could contribute substantially to reducing quill infectivity. The quills of the North American porcupine remain formidable defense weapons, but their efficacy rests on physical factors, not microbiological ones.

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BRANCH-CUTTING BEHAVIOR BY THE VOLE (*Microtus pennsylvanicus*) A Mechanism to Decrease Toxicity of Secondary Metabolites in Conifers

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Abstract—While investigating the preferences of meadow voles (*Microtus pennsylvanicus*) toward various coniferous species, we noted they often cut the branches of young trees, and then left them lying on the snow for two days or more before eating them completely. We tested whether this "behavioral manipulation" of a potential food resource resulted in a decrease in the level of secondary metabolites in branches. We found that after being cut for more than three days, the branches of young conifers had significantly less phenolics and condensed tannins. These postmanipulation levels of secondary metabolites were similar to those found in preferred summer food resources. Voles were thus capable of transforming a low-quality resource into one that was more compatible with their feeding patterns and detoxification mechanisms.

Key Words—Meadow vole, *Microtus pennsylvanicus*, plant–animal interaction, conifers, phenolics, tannins.

INTRODUCTION

Herbivores have developed different means to reduce the toxicity of secondary metabolites commonly found in plants (Levin, 1976). Freeland and Janzen (1974) have suggested that mammals could transform terpenes into vitamin A, E, and K precursors, but the most striking adaptations reside in the food manipulation behaviors that many species have developed. African ungulates are known to prefer tree species that are low in tannins (Cooper and Owen-Smith, 1985) while Colobus monkeys (*Colobus quereza*) prefer the foliage of trees that

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have low levels of procyanidin tannins (Oates et al., 1977). Mountain hares (*Lepus timidus*) and snowshoe hares (*Lepus americanus*) are so sensitive to triterpenes or phenolic glycosides that they select very carefully individual trees or parts of trees having the lowest concentration (Reichardt et al., 1984; Tahvanainen et al., 1985). Large herbivores, like smaller ones, are thus well equipped to evaluate the palatability of potential foods on the basis of phenolic compounds (Beart et al., 1985; Swain, 1979). While doing cafeteria tests with meadow voles (*Microtus pennsylvanicus*) to establish their ranking preferences toward various coniferous species, we observed that they routinely manipulated the conifers in a peculiar way. After cutting the branches from the trunk and stripping the needles from the branches, they left the branches on the snow for two or three days before eating them completely. We set up an experiment mimicking this behavior to find out whether it had an effect on the levels of secondary metabolites in branch tissues. The target metabolites were phenolics and tannins, because they are already known to affect food selection, growth, and survival in the vole (Lindroth et al., 1986; Bergeron and Jodoin, 1987).

METHODS AND MATERIALS

The following procedure was adopted to mimic exactly the branch-cutting behavior that voles showed in cafeteria tests during winter. On day 1, all branches of 30 young conifers were cut off and left exposed outdoors to early winter conditions. The enclosure was adjacent to the animal house and roofed to protect the experimental animals from rain, sleet, and snow. Ambient temperature ranged from -5 to $0°C$ at the time of study. The branches of 10 more trees per species were cut, stripped of their needles, and brought to the laboratory to be divided into three subsamples for immediate analysis. The first subsample consisted of the whole branch; the others were comprised of bark and cambium and of secondary xylem only. These samples were ground to 1 mm in a Brinkmann mill and processed immediately. On day 3, needles of the 30 trees left outside were removed from the branches, and a sample involving 10 of these trees was brought to the laboratory to be divided into another three subsamples to be processed as previously described. On day 5, all branches of the remaining 20 trees were dissected to separate bark and cambium from the secondary xylem, and a sample of 10 of these trees was brought to the laboratory for immediate analysis. The remaining material from the last 10 trees was analyzed on day 7 to complete the time gradient observed from voles.

Manipulations of young trees ($3\frac{1}{2}$ years; height 30–45 cm) were all performed under winter conditions, and the samples were ground and analyzed the same day to circumvent the rapid chemical changes in the laboratory that characterize phenolics and tannins in biological material (Lindroth and Pajutee, 1987). Total phenolics were estimated from the colorimetric method of Single-

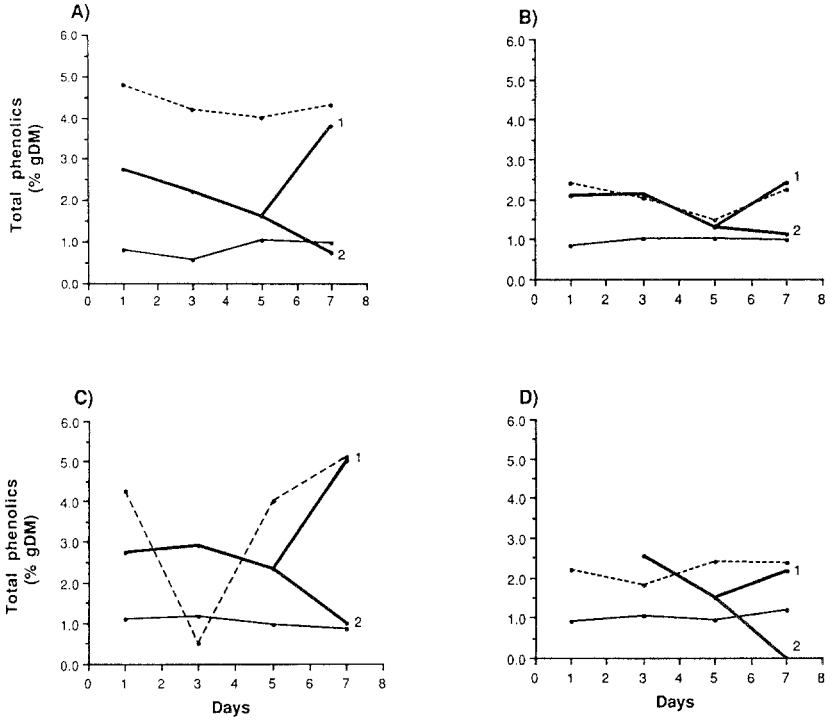

FIG. 1. Estimation of total phenolics in branch tissues of various coniferous species: ———— whole branch, ---------- bark/cambium layer, ———— xylem. (A) white spruce, (B) Norway pine, (C) Norway spruce, (D) white pine. Note division of the whole branch after five days into two subsamples, the bark-cambium (1) and xylem tissues (2).

ton and Rossi (1966) using gallic acid as a standard (readings at 765 nm). Condensed tannins were quantified following the colorimetric procedure of Burns (1971) as modified by Price et al. (1978) using a 1 : 1 mixture of catechin and epicatechin as standards (readings at 500 nm). Experiments were performed on duplicate samples of four coniferous species: Norway spruce (*Picea abies*), white spruce (*Picea glauca*), Norway pine (*Pinus resinosa*) and white pine (*Pinus strobus*). Differences in phenolic or tannin levels between day 1 and day 5 were drawn from one-way ANOVAs (SAS Institute, 1985).

RESULTS

Phenolic contents in whole branches tested decreased dramatically from day 1 to day 5 for three of the four coniferous species (Figure 1). In white spruce, phenolics dropped from 2.8%/g DM (Dry Matter) to 1.5% on day 5

(Figure 1A), which represents a significant loss ($Fs = 122.09$, $P < 0.05$) of 72%. Branches of Norway pine (Figure 1B) showed a loss of 64% ($Fs = 63.73$, $P < 0.05$), while those of Norway spruce (Figure 1C) yielded the lowest, albeit significant, results with a 22% decline ($Fs = 18.08$, $P < 0.05$). Dosages in branches of white pine (Figure 1D) could not be established on day 1 because of a handling error, but a closer examination of phenolics found in the bark–cambium and xylem tissues suggests that similar phenomena could also exist for the species. Total phenols increase slightly in three of the four coniferous species between day 5 and 7 to dosages that become almost equal to those of day 1.

The reduction of condensed tannins in branches follow exactly the patterns showed by phenolics (Figure 2). In white spruce (Figure 2A), doses varied from 0.29%/g DM at day 1 to 0.18%/g DM at day 5, representing a loss of 68% ($Fs = \infty$, $P < 0.05$). Branches of Norway pine (Figure 2B) lost 60% of their

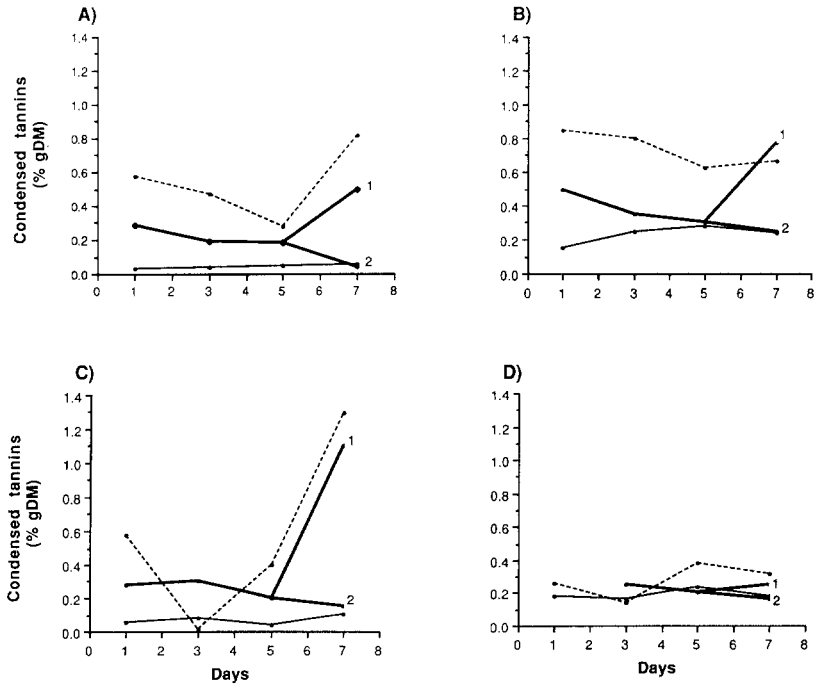

FIG. 2. Estimation of condensed tannins in branch tissues of various coniferous species: ——— whole branch, ---------- bark/cambium layer, ——— xylem. (A) white spruce, (B) Norway pine, (C) Norway spruce, (D) white pine. Note division of the whole branch after five days into two subsamples, the bark–cambium (1) and xylem tissues (2).

contents in tannins during the same period ($Fs = 70.0$, $P < 0.05$), while that of Norway spruce (Figure 2C) decreased by 43% ($Fs = 16.0$, $P < 0.07$). Branches of white pine (Figure 2D) would probably have followed the same pattern because tannin levels in bark–cambium and xylem subsamples vary in the same way as the other species (Figure 2).

DISCUSSION

Voles are well known to cause debarking damage to deciduous and coniferous trees in winter (Sartz, 1970; Byers, 1974; Wysolmerski et al., 1980). Reports in the literature cite that they gnaw partly or entirely the base of trunks (Jokela and Lorenz, 1959), and more rarely the branches, although Hansson (1985) reported this type of feeding behavior. However, voles cannot cut the branches off trees very easily unless thay are young trees or trees exposed above the snow cover.

We had the opportunity in the spring of 1988, after a peak year of vole numbers, to observe more than 10 million young coniferous trees destroyed in a government nursery using the branch-cutting method that we have just explored in this experiment. Herbivores are known to prefer high-nutrient food resources with low levels of secondary metabolites such as polyphenolics (Bryant, 1981; Bryant and Kuropat, 1981; Sinclair et al., 1982; Palo, 1984; Tahvanainen et al., 1985). Hares seem to be able to detect camphor dosages in conifers, notably in white spruce, and adjust their browsing behavior accordingly (Sinclair et al., 1988). Furthermore, food preferences of meadow voles are positively associated with low phenolic contents in plants (Bergeron and Jodoin, 1987). We have shown elsewhere (Bergeron and Jodoin, 1987) that unpreferred herbaceous species contained about twice as much phenolic (± 3.0 to 5.0%/g DM) compared with preferred species (± 1.3 to 2.5%/g DM). Moreover, it is known that voles grow poorly and have poor body condition when fed diets with phenolic additives (Lindroth et al., 1986). Condensed tannins have also been associated with low reproductive success (Berger et al., 1977) and reduced food intake (Lindroth and Batzli, 1984) in many vole species. In spite of the fact that most of the phenolics tested were different in structure, we can very easily see the reasons why voles can readily detect them in plants.

This experiment showed that phenolics from fresh-cut branches of most conifers were as high as those found in avoided plant resources of a vole habitat. We argue here that voles do not have to use detoxification mechanisms to transform a low-quality food into a potentially good food resource. By cutting the branches and letting them stand under normal early winter conditions, voles reduced by half the doses and transformed on a short-term basis (within five days) the twigs into usable food. Disappearance of phenols may be explained

in terms of polymerization or oxidation processes that have not been followed in this experiment. Isoprenoids are volatile compounds that could have disappeared easily after handling by voles. The sudden increase of phenols after the initial five-day period is unexplainable at the present time but suggests that voles must use quickly such food resources to maximize nutrient intake. Samples of cut twigs yielded protein contents as high as 12% in winter (Bergeron, unpublished data). If this is coupled with low amounts of phenolics (1.5%/g DM), this material can be regarded as a high-quality food supply. Triggering mechanisms leading voles to switch from their normal winter diet in terms of stems, roots, fruits, and seeds of herbaceous species (Batzli, 1985) to young deciduous or coniferous trees are not understood at present time, but whatever the cause, these animals have developed a remarkable adaptation that may increase tremendously the overall food supply in a harsh season.

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A NEW SARPEDOBILIN-CONTAINING BUTTERFLY *Papilio graphium stresemani stresemani* and Its Bioecological Situation Within the Species

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Abstract—The doubly cyclized bile pigment sarpedobilin (**3**) represents a unique case in nature. In spite of being present in trace amounts in many butterfly and moth species containing pterobilin (**1**) or phorcabilin (**2**) as the main bilin, this substance occurs as the main pigment in a restricted number of species geographically limited to Southeast Asia. We have isolated and identified sarpedobilin **3** (as the dimethyl ester) as the main bile pigment from *Papilio graphium stresemani stresemani* (from Ceram island, Indonesia) in the amount of ca. 0.1 mg per individual, double that found in *Papilio sarpedon* or *P. weiskei*. We conclude that *P. sarpedon* (and subspecies) *P. weiskei,* and *P. stresemani* represent, in terms of evolution (see article), a group of closely related species. By comparison of colored wing patterns and resistance of the chromoproteins to hydrolysis by MeOH–H_2SO_4, *P. stresemani* is near *P. weiskei*. The bioecological relationships of the three sarpedobilin-containing species is discussed.

Key Words—Sarpedobilin, bile pigment, butterfly, *Papilio graphium stresemani stresemani, P. sarpedon, P. weiskei,* Lepidoptera, Papilionidae, bioecological relationships.

INTRODUCTION

We have shown, over the past 25 years (Barbier, 1981) that the linear bile pigment pterobilin (biliverdin IX γ, **1**, Figure 1) is widely distributed among both larvae and adults of butterfly and moth species. This screening of the Lepidoptera was extended to some 250 species and led to the isolation of two other blue–green bilins, phorcabilin (**2**) and sarpedobilin (**3**), pigments which were first extracted from *Papilio phorcas* (males) and *Papilio sarpedon* (Vuillaume and Barbier, 1969; Choussy and Barbier, 1975; Bois-Choussy and Barbier,

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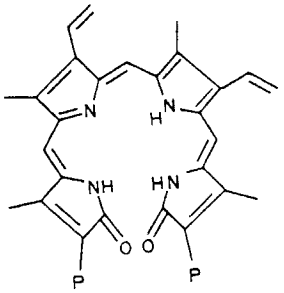

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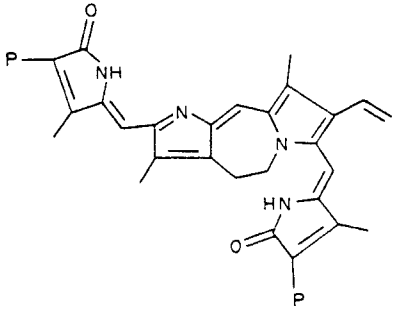

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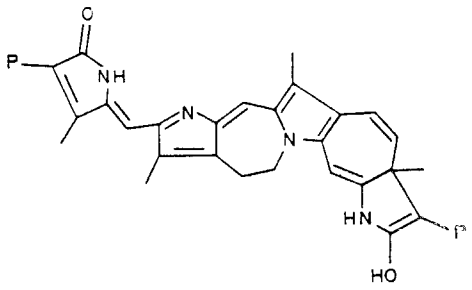

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FIG. 1. Butterfly and moth bile pigments: (**1**) pterobilin, (**2**) phorcabilin, (**3**) sarpedobilin. P = $-CH_2CH_2COOH$ or $-CH_2CH_2COOCH_3$ in the methyl esters.

1977, 1978). Pterobilin **1** is formed from the ring opening of protoporphyrin IX at the γ bridge position. Phorcabilin **2** and sarpedobilin **3** can be obtained in vitro by irradiation with visible light (Choussy and Barbier, 1976; Bois-Choussy and Barbier, 1978). These substances occur in the hemolymph or in the cuticle and wing membranes, and are bound to proteins that protect them from irradiation. Chromoproteins of **1** and **2** cannot be photocyclized as shown by an in vitro experiment (Bois-Choussy, 1977; Barbier, 1981). Many species contain trace amounts of phorcabilin and sarpedobilin as secondary bile pigments (Barbier, 1981). Species containing sarpedobilin **3** as the main pigment are very rare and, until now, where limited to *P. graphium sarpedon* (all investigated subspecies to date) and to *Papilio weiskei* (Choussy and Barbier, 1975). *P. sarpedon* is widely distributed in the southeast of Asia, while *P. weiskei* is more abundant in Papua and New Guinea. *P. weiskei* shows an extraordinary patchwork of colors on its wings with superimposition of zones containing sarpedobilin (blue-green) with pink areas from which ommin was isolated (Barbier, 1983). Recently, the screening among species was extended to butterflies and moths from South America, but we were unable to discover any other sarpedobilin-containing species. This observation pointed out the scarcity of Lepidoptera containing this pigment. Due to its polycyclized structure, sarpedobilin **3** is more reminiscent of an alkaloid than of a bile pigment, and it was shown to give stable adducts with polyanions. Thus, dicarboxylic acids of the Krebs cycle (Barbier, 1985), ATP, and DNA, react with sarpedobilin (as the dimethyl ester), and this reactivity explains the moderate growth-inhibiting properties (Vuillaume et al., 1986) that were noted in vitro.

Recently we noted a rare butterfly species, *Papilio graphium stresemani stresemani*, presenting blue-green spots on the wings with a pattern that resembles *P. weiskei* but with no apparent pink areas, and we decided to extract and study this pigment. We present the isolation, identification, and spectrophotometric determination of sarpedobilin in this species and discuss the bioecological relationships involved.

METHODS AND MATERIALS

Wings of *Papilio graphium stresemani stresemani* from Ceram Island, Moluccas Archipelago, Indonesia, were cut into small pieces and submerged in a mixture of absolute methanol–sulfuric acid 15% (ca. 4 ml/animal). The suspension was stirred vigorously with a magnetic stirrer to a paste and then kept 30 hr at room temperature. After filtration through a small cotton plug and washing the filter by ca. 2 vol of methylene chloride (8 ml/animal), twice the volume of water was added. After shaking, the organic CH_2Cl_2 phase contained all the pigment dimethyl ester. The organic phase was washed twice with an equal volume of water, dried over Na_2SO_4, and concentrated to a small volume prior to chromatography.

TLC was carried out on Schleicher-Schüll SiO_2 films, developed with a mixture of CCl_4–CH_2Cl_2–MeOH 5 : 1 : 1 (R_f of sarpedobilin dimethyl ester, 0.35), and compared with a standard. Authentic samples of pterobilin, phorcabilin, and sarpedobilin dimethyl esters for comparison were prepared according to Bois-Choussy and Barbier (1983) or extracted from butterflies (*Pieris brassicae, P. phorcas*, and *P. sarpedon*). Spectrophotometric determination of the pigment dimethyl ester was carried out on a Perkin-Elmer lambda-5 automatic recorder. Microdegradation of the pigment dimethyl ester was obtained by the action of chromic acid according to Rüdiger et al., 1968, and the fragmentation pattern was compared with an authentic sample of sarpedobilin dimethyl ester (TLC, development with CCl_4–cyclohexane–ethyl acetate 5 : 1 : 3), visualization of imides by treatment with Cl_2, and spraying with a benzidine solution.

RESULTS AND DISCUSSION

Papilio graphium stresemani stresemani presents on its wings a series of green and deep blue spots in a patchwork similar to that of *P. weiskei*, except for the pink areas due to accumulation of ommin. *P. graphium stresemani stresemani* is a rare butterfly occurring in Ceram Island, Indonesia, that is, in a very delimited geographical zone. Extraction of the bile pigment as the dimethyl ester was accomplished by treatment of the butterfly wings with methanol–sulfuric acid according to Choussy and Barbier (1975). Identity with sarpedobilin dimethyl ester was demonstrated by direct comparison with an authentic sample isolated from *P. sarpedon* by using the same method (cochromatography on SiO_2 layer with CCl_4–CH_2Cl_2–MeOH 5 : 1 : 1, R_f = 0.35), VIS spectra in methanol (single maximum at 600 nm), shift of the maximum of absorption to 660 nm by adding malonic acid (Barbier, 1985) (formation of an adduct, a specific property of sarpedobilin), and chromic acid oxidation to imides as reported (Choussy and Barbier, 1975).

The spectrophotometric determination of the pigment after isolation indicated ca. 0.1 mg/animal, that is to say approximately double the amount of sarpedobilin dimethyl ester previously isolated from *P. sarpedon* or *P. weiskei* obtained from Malaya and New Guinea.

Biosynthesis of the bilins in butterflies and moths appears to be under genetic control, resulting in specificity in the structure of the final main pigment. Due to its insularity, *P. stresemani* has very likely undergone genetic changes from a common ancestor with *P. weiskei*, both species remaining near neighbors of *P. sarpedon*, which had a wider distribution. These three sarpedobilin-containing species thus form a bioecological group of closely related butterflies that have followed different paths of evolution.

Several attempts to establish the role of irradiation in the transformation of pterobilin into sarpedobilin in vivo led to contradictory results. *P. sarpedon* larvae were reared in absence of light (except for cleaning and feeding), and sarpedobilin dimethyl ester was spectrophotometrically determined. As could be expected from such experiments, mortality among the larvae reared under such conditions from L_1 to L_5 was very high, and results were not reproducible in four different assays. In these experiments, the amount of sarpedobilin in the surviving pupae was 65% more in the first series, but 30, 48, 60% less in three further assays, by reference to rearing in normal condition of light. For example, in the fourth experiment, of 60 larvae that were reared in absence of light from L_2 to L_5, only eight survived to pupation and contained ca. 10–12 μg each of sarpedobilin, while standard pupae contained 18–22 μg of pigment. It is, of course, impossible to draw conclusions from these experiments carried out under difficult biological conditions. It thus remains to be learned if the cyclization of pterobilin **1** into phorcabilin **2** and sarpedobilin **3** is due to the action of light on the integuments or if these cyclized bile pigments arise from an enzymatic process. A relay should be taken by biologists to demonstrate if, by means of cellular extracts, the cyclized bilins **2** and **3** could be formed from pterobilin in the absence of light.

When the extraction of sarpedobilin is carried out on the wings of butterflies that were simply cut into pieces, not mashed with a magnetic stirrer, great differences are noted between the species. *P. sarpedon* gives most of the pigment extracted into the methanol–sulfuric mixture after 30 hr, while *P. weiskei* only furnishes a small amount. In the case of *P. stresemani*, nearly all the pigment remains unextracted from the wing membrane. This last case seems to indicate a similarity with *P. weiskei*, which is susceptible to a biogenetic interpretation. It has been shown previously that these pigments are bound as chromoproteins within the wing membranes. [They can be extracted as such by a phosphate buffer, pH 7.2, and purified by repeated precipitations with 25, 20, 15% ammonium sulfate solutions, according to Bois-Choussy (1977).] The biosynthesis of these associated proteins and their binding to the wing membranes are, of course, under genetic controls. Due to the small number of animals so far available, such chromoproteins from *P. stresemani* could not be extracted and chromatographically compared.

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BEHAVIORAL RESPONSE OF MALE WHITE PEACH SCALE TO THE SEX PHEROMONE, (*R*,*Z*)-3,9,-DIMETHYL-6-ISOPROPENYL-3,9-DECADIEN-1-OL PROPIONATE AND CORRESPONDING ALCOHOL[1]

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Abstract—Males of the white peach scale, *Pseudaulacaspis pentagona* (Targioni-Tozzetti), in Florida differ from this scale in France in their responses to putative pheromonal chemicals presented in a laboratory bioassay. Males from Florida were attracted to, and exhibited sexual (copulatory) behaviors at, the locus of evaporation of (*R*,*Z*)-3,9-dimethyl-6-isopropenyl-3,9-decadien-1-ol propionate (P) (previously identified from effluvia of Florida females). Addition of the corresponding primary alcohol (A) to the propionate did not alter the attraction of Florida males to the synthetic P and did not alter their sexual responses from those exhibited by males exposed to P alone. In contrast, the effluvia from French scales has been reported to contain P and A, and the alcohol has been reported in laboratory studies to be required to release sexual behavior in French males. This suggests that the conspecific status of the scale in Florida and France should be reexamined. Field trap baits with 2.5 μg of a 1 : 1 mixture of P and A captured significantly fewer Florida males than traps baited with 1.25 μg of P alone, but traps similarly baited with 0.125 μg of each material captured the same number of insects as traps baited with 0.125 μg of P alone.

Key Words—Pheromonal races, white peach sale, *Pseudaulacaspis pentagona*, Homoptera, Coccoidea.

[1] Mention of a commerical or proprietary product in this paper does not constitute an endorsement of that product by the USDA.

INTRODUCTION

A volatile chemical, (*R*,*Z*)-3,9-dimethyl-6-isopropenyl-3,9-decadien-1-ol propionate, was identified by Heath et al. (1979) as a female sex pheromone of the white peach scale, *Pseudaulacaspis pentagona* (Targioni-Tozzetti) in Florida, U.S.A. Sex pheromones are commonly blends of chemicals, and Einhorn et al. (1983) reported the presence of the above propionate and the corresponding alcohol in volatile material emanating from female white peach scales in France. They described laboratory behavioral assays (Einhorn et al., 1983) in which the propionate attracted males while the alcohol did not; however, very few of the males exposed to only the propionate exhibited sexual behavior. When males were exposed to mixtures of the alcohol and propionate, many of them were attracted and displayed sexual behavior. Conversely, Heath et al. (1979) (assays conducted by J.R.M.) had reported that U.S. males responding in a similar assay to the biosynthesized propionate obtained from extracts of female volatiles or to laboratory-synthesized propionate exhibited sexual behavior that appeared the same as that of males exposed to pheromone-releasing females.

This study examines the response of U.S. males to these putative pheromonal chemicals (synthetic) and to the natural propionate and alcohol isolated and purified from extracts from French females.

METHODS AND MATERIALS

The chemicals were synthesized and analyzed according to the methods described in Heath et al. (1979, 1980) ($\geq 99\%$ *R* and $\geq 98\%$ *Z*).

Volatiles from French scale were provided by J. Einhorn, CNRS Laboratoire des Médiateurs Chimiques, Domain de Brouessy, Magny-les-Hameaux, 78470 Saint-Rémy-lés-Chevreuse, France. French scale was collected from peach and mulberry and reared on white (Irish) potato. The volatiles were collected as described by Einhorn et al. (1983) and were dissolved in pentane. The propionate and alcohol within these volatile extracts were isolated on a silica column and by gas–liquid chromatographic methods described in Heath et al. (1979, 1980) and analyzed in the manner described by Heath et al. (1979, 1980) using capillary column chromatography (Dexsil 300 GC, 60 m × 0.76 mm ID; OV-101, 35 m × 0.25 mm ID; SP-1000, 45 cm × 0.25 mm ID), and GC-MS (Nermag R-10-10 C in EI and CI modes). The isolated materials were at least 99% pure. The naturally derived alcohol and propionate were recombined in pentane in a ratio of 1 : 1. This ratio was chosen because it is in the mid-range of the ratios tested by Einhorn et al. (1983).

The insects used in the laboratory bioassay were newly emerged from pupae on infested peach wood obtained at Quincy, Florida. The assays were con-

ducted during the afternoon hours of peak male emergence (1–4 PM) (McLaughlin and Ashley, 1977). The procedure was that described in Heath et al. (1979). Groups of males were aspirated into small clear glass bottles. A test sample in 5 μl of pentane was then placed on an 8 × 12-mm stainless-steel planchet at the center of a 90-mm-diam. arena (inverted plastic Petri dish lid lined with filter paper). After the solvent evaporated, males (mean = 11.4, range 6–16) were introduced ca. 25–30 mm from the treated end of the planchet by sharply striking the lip of the open bottle mouth against the floor of the arena. The cover (dish bottom) was placed on the arena and the males were observed for 2 min. (The arena covers were wiped with a 10% solution of ethylene glycol in water to reduce the static charge, which would interfere with the mobility of the males.) The number of males on or touching the planchet, the number exhibiting copulatory movements, and the total number of males in the arena were recorded at the end of the 2-min observation period. An arena was used only once in a day. The filter paper liner was discarded, and the Petri dish was washed in a water–detergent solution and stacked open to dry and air out for three days before being used again. The planchets were washed with pentane and held overnight in a 100°C oven. Each treatment was tested concurrently in three different arenas each day (replicate). Tests were conducted on 10 different days at doses of 10 pg, 1 pg, and 0.1 pg of synthetic propionate alone and of synthetic alcohol to propionate ratios of 6 : 1, 1 : 1, and 0.1 : 1, in which the dose of propionate was 10 pg, 1 pg, and 0.1 pg. A test also was conducted with the natural propionate and alcohol obtained from the French scale extract (1 : 1 ratio) at the same three doses and number of replications.

Field assays were conducted in a peach orchard (13 ha) near Hawthorne, Florida. Each field trap consisted of a 22 × 14-cm panel constructed from the cardboard top of a Pherocon 1C-type trap (Trece, Inc., Salinas, California) covered on both faces with an insect trapping material (Stickem Special, Michel & Pelton, Co., Emeryville, California) mixed with about 10% (volume) of mineral oil. A bait was treated with the candidate attractant and suspended within a 2 × 2-cm opening cut at the center of each sticky panel. The baits were prepared from cylindrical 8-mm × 3-mm-diam. needle seal rubber septa (Chemical Research Supplies, Inc., Addison, Illinois) which were cut lengthwise into quarters. Each material or mixture was dissolved in 10 μl of pentane and pipetted onto a cut septum. The treated septa were held in a laboratory hood at about 23°C for two days prior to being placed in traps.

The traps were suspended 1.5 m from the ground and 30 cm apart in a line. Each trap line was about 3 m from the nearest scale-infested tree. For each experiment, each treatment was assigned a random location within a trap line (block) and then systematically moved to the adjacent position each time the traps were inspected. Two blocks of traps, separated by 50–80 m, were employed in each experiment. The first experiment compared the capture of

males on traps baited with 1.25 μg of synthetic propionate, 2.5 μg of the 1 : 1 mixture of female-synthesized P and A, and pentane controls. These traps were inspected on eight consecutive days. The bait was transferred each day to a clean panel and moved to the next trapping position. The panels were brought into the laboratory and the male captures determined and recorded. In the second experiment, the traps were baited with two doses (1.25 or 0.125 μg) of synthetic propionate alone, synthetic alcohol alone, a 1 : 1 mixture of synthetic alcohol–propionate, or a pentane control. The second test was conducted for six days.

RESULTS

Males responded to pheromone in the bioassay by rapidly walking directly to or in an arc toward the treated planchet. Males never walked or flew onto untreated steel planchets placed in assay dishes. I have observed several hundred males of white peach scale interacting with colonies of sexually mature females on potato or collected peach wood and on peach trees. Males that fly to a female or colony of females land adjacent (millimeters to 1–2 cm) to a female and walk to her. Typically, a male mounts the cover of the female, turns to orient to her (head toward the center of the cover and genital stylet aligned with the pygidium of the female), and exhibits a very characteristic probing behavior under the lip of the cover with his aedeagus. These same behaviors are released in the presence of female sex pheromones in the laboratory assay: The male walks to the locus of the evaporating pheromone, mounts the planchet and then rocks back and forth while thrusting at the surface between its metathoracic legs with its aedeagus, often turning as if attempting to become properly oriented to a female.

Behaviors that resulted in males arriving upon the planchet are termed attraction, and behaviors involving thrusts with the aedeagus are termed copulatory behavior in the ensuing discussion. These behaviors were performed by the Florida males in the presence of the synthetic propionate alone, and the addition of the alcohol did not alter this response of U.S. males.

Table 1 summarizes the attraction and copulatory behavior measured in the laboratory bioassays. The attraction responses are in very close agreement with the data of Heath et al. (1979). Copulatory behavior was noted with all doses of propionate alone, and neither attraction nor copulatory behavior appeared to be altered by the presence of the alcohol in mixtures with the propionate, i.e., mean percentages for males attracted (or exhibiting copulatory behavior) at each dose are not statistically different across propionate–alcohol mixtures (Table 1). Neither attraction nor copulatory behavior was exhibited in tests with the alcohol alone. Both behaviors were reduced in the 6 : 1 (A/P) treatments, which may reflect a reduction in the evaporation rate of the propionate but does not

TABLE 1. RESPONSE OF U.S. MALE WHITE PEACH SCALES TO SYNTHETIC AND FEMALE-PRODUCED (*R*, *Z*)-3,9-DIMETHYL-6-ISOPROPENYL-3,9-DECADIEN-1-OL (A) AND CORRESPONDING PROPIONATE (P) IN LABORATORY BIOASSAY[a]

	Dose (pg) of propionate applied to planchet[b]		
Ratio A/P	10	1	0.1
0:1	83 ± 5 (96)	52 ± 5 (84)	39 ± 6 (69)
0.1:1	79 ± 6 (98)	49 ± 4 (88)	37 ± 8 (61)
1:1	84 ± 9 (96)	55 ± 4 (90)	39 ± 8 (71)
1:1 (female)	82 ± 11 (96)	53 ± 8 (91)	39 ± 10 (71)
6:1	74 ± 8 (95)	45 ± 6 (83)	30 ± 8 (60)
1:0	00	00	00

[a] Mean percentage ± SEM of males attracted to the planchet and (mean percentage of attracted males exhibiting sexual behavior).
[b] Mean percentages (attraction or sexual behavior) within columns for mixtures containing the propionate are not statistically different, Student-Newman-Keuls multiple range test ($P = 0.05$) (SAS Institute, 1985).

appear to indicate any strong inhibitory effects due to the alcohol. Responses to the 1:1 mixture of natural alcohol and propionate were equivalent to those of the synthetic materials at all doses.

The results of the field-trapping experiments are summarized in Tables 2 and 3. The 1:1 mixture of natural propionate–alcohol captured only 49% of the number of males captured by the bait containing only the synthetic propionate (Table 2). When the synthetic alcohol was added (1:1) to 1.25 μg of the synthetic propionate, male captures were reduced by 71% (Table 3). However,

TABLE 2. MEAN NUMBER OF U.S. MALE WHITE PEACH SCALE CAPTURED PER DAY PER TRAP BAITED WITH FEMALE-SYNTHESIZED (*R*, *Z*)-3,9-DIMETHYL-6-ISOPROPENYL-3,9-DECADIEN-1-OL (A) IN MIXTURE WITH CORRESPONDING PROPIONATE (P) OR LABORATORY-SYNTHESIZED PROPIONATE

Treatment and amount (μg)	Number replicates (2 traps/replicate)	Mean capture[a] ± SEM (per day/trap)
Synthetic propionate (1.25)	8	84.1 ± 12.1
Female-synthesized (1.25 A–1.25 P)	8	41.4 ± 5.0
Pentane control	8	22.2 ± 2.1

[a] Means all differ significantly ($P = 0.05$). Student-Newman-Keuls multiple range test (SAS Institute, 1985).

TABLE 3. MEAN NUMBER OF MALE WHITE PEACH SCALE CAPTURED IN TRAPS BAITED WITH SYNTHETIC (*R*, *Z*)-3,9-DIMETHYL-6-ISOPROPENYL-3,9-DECADIEN-1-OL AND CORRESPONDING PROPIONATE

Material and amount (μg)	Number replicates (2 traps/replicate)	Mean capture[a] ± SE (per day/trap)
Propionate (1.25)	6	1,531.2 ± 159.3 a
Propionate-alcohol (1.25 each)	6	445.5 ± 39.6 b
Propionate (0.125)	6	126.3 ± 9.2 c
Propionate-alcohol (0.125 each)	6	134.0 ± 12.9 c
Alcohol (1.25)	6	66.5 ± 5.2 d
Alcohol (0.125)	6	42.2 ± 4.5 d
Pentane control	6	63.4 ± 5.9 d

[a] Means followed by different letters are significantly different ($p = 0.05$). Student-Newman-Keuls multiple range test (SAS Institute, 1985).

when the dose of the propionate and alcohol was reduced 10-fold, the propionate and the 1 : 1 (P/A) mixture captured like numbers of males (Table 3). Male captures with the alcohol alone were not statistically different from captures in the unbaited control traps.

DISCUSSION

The alcohol at the higher dosage reduced the number of males captured in field traps, but it did not reduce the response of males to a locus of evaporating propionate in the laboratory bioassay. This may have been due to the presumably higher evaporation rate of the alcohol from the trap bait (1.25 μg) than from the assay planchet. Certainly, the laboratory assay and field trap data in which the lesser dose of alcohol was presented (0.125 μg) suggest that the alcohol is not highly inhibitory to male attraction.

Heath et al. (1979) found no evidence for the presence of behaviorally active chemicals other than the propionate in Florida scale and did not analyze fractions of the effluvia that may have contained the alcohol. Their identification was based upon the laboratory assay described here, which, in the case of the Florida scale, is not influenced by the alcohol within the dosages tested. Thus, the presence of the alcohol in the volatile emissions of sexually mature Florida scales is not precluded.

Einhorn et al. (1983 and personal communication) reported that the volatile emissions of the French female white peach scales contained 15–40% of the propionate and 60–85% of the alcohol. A mixture of 1 : 1 alcohol to propionate

would evaporate the two compounds at a ratio (alcohol–propionate) of about 80:20 [estimated from gas chromatography data using the method of Heath et al. (1986)]. Einhorn et al. (1983) tested dose ratios (amount applied to the evaporative substrate) of 500:85, 50:8.5, and 85:85 (ng of alcohol–ng of propionate) in their laboratory assay, which was similar to the assay I described in Heath et al. (1979) and in this study. These are much greater amounts than were applied in assays with Florida scales; however, the French assays were conducted using filter paper (Whatman No. 3) as the evaporative substrate (Einhorn et al., 1983) while the Florida assays utilized a metal surface. Experiments (McLaughlin, unpublished) leading to the development of the assay described in Heath et al. (1979) determined that the amounts of female extracts placed on filter paper that elicited a given level of male response were greater than the amounts required when the steel surface was used (these preliminary tests indicated at least a 10- to 100-fold difference). This difference in bioassay method prevents any definitive conclusion regarding the relative sensitivity of French and U.S. males to pheromone in laboratory bioassay; however, when taken together, the results of this paper and of the experiment of Einhorn et al. (1983) strongly indicate that U.S. males from Florida and French males respond differently to the propionate in the absence of the alcohol.

These results suggest that the taxonomic status of this species should be reexamined. White peach scale occurs worldwide and has a very great host range. The suggestion that there are different races or forms of *P. pentagona* has been put forward in several reports of differences in egg coloration (Tremblay, 1958; see also Bennett and Brown, 1958) and variation in host relationships and parasites (for some examples see Hughes, 1960; Clausen, 1978; Cock, 1983). One taxonomic revision in the United States resulted in the recognition of an additional cryptic species, *P. prunicola* (Maskell) (Davidson et al., 1983) whose sex pheromone has not been examined.

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POSSIBLE ROLES OF COTTON BUD SUGARS AND TERPENOIDS IN OVIPOSITION BY THE BOLL WEEVIL

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Abstract—Several cotton *Gossypium* spp. race stocks have been identified that possess resistance to the boll weevil *Anthonomus grandis* Boh. because oviposition is decreased. In this work, a number of known cotton constituents that influence stimulation of feeding and attractancy for this insect were found to have little or no influence on oviposition. These include gossypol, β-bisabolol, caryophyllene, some fatty acids and their methyl esters, some wax esters, flavonoids, condensed tannins, and chrysanthemin. Analysis of cotton bud surfaces showed that the content of volatile terpenoids was generally higher in resistant lines, but bioassays did not show decreased oviposition in the presence of the terpenoids. The sugars (glucose, fructose, and sucrose) found in anthers, uniformly stimulated oviposition in the bioassay, and their content was higher in susceptible lines. These results suggest that a major basis of resistance to boll weevils as related to oviposition may be the decreased content of sugars in resistant lines. The analysis of free sugars in the anthers, and perhaps also the analysis of bud surface terpenoids, may provide a basis for selection or genetic production of cotton lines resistant to the boll weevil.

Key Words—Cotton, *Gossypium* spp., boll weevil, *Anthonomus grandis*, Coleoptera, Curculionidae, oviposition, plant–insect interaction, terpenoids, sugars.

INTRODUCTION

The identification of constituents in cotton *Gossypium* spp., plants that affect boll weevil *Anthonomus grandis* Boh. (Coleoptera: Curculonidae) feeding and oviposition behavior has been pursued over the past 30 years since the report of Keller et al. (1962) that feeding stimulants were present in cotton flower

buds. Reports on the constituents of flower buds were made by Chan et al. (1978); Hanny (1980); Hedin et al. (1967, 1977); Struck et al. (1968a,b); Waiss et al. (1981). McKibben et al. (1985) recently identified some cotton plant wax esters with feeding stimulant activity. Since the 1960's, there also has been an active interest in cotton volatiles that affect boll weevil behavior, particularly attractancy. However, factors affecting boll weevil oviposition behavior have not been similarly addressed, although it was observed that certain primitive (wild) race stocks suppressed oviposition. Jenkins et al. (1978) reported that oviposition was decreased to 30% and 36% of normal in field tests with two selected entries, T-326 and T-1180. In later no-choice laboratory tests, McCarty et al. (1987) showed that while feeding punctures were 24% higher in flower buds of these two primitive race stock entries, presumably because of changes in searching and probing behavior, oviposition was reduced to 54% and 57% of the control. In a subsequent field test, damaged squares were reduced to 59% and 65% of the control and adult emergence was decreased to 47% and 50% of the control (McCarty et al., 1987). These studies indicate selection for host-plant resistance to the boll weevil may be possible, and the resistance may have a chemical basis.

The adult female moves to the flower bud after alighting on the plant and then proceeds to construct an oviposition hole in which the egg is placed and the hole sealed. It is not understood how chemicals and/or other factors may affect the normal behavior of the female in making a decision to oviposit. Bud surface chemicals may modulate initial behavioral responses. Chemicals in the carpel and anthers that are encountered in the excavation of the hole may also affect the oviposition sequence. In this present study, we report efforts to establish laboratory bioassays that will reliably evaluate the constituents that the female encounters both on the bud surface and in the anther that affect the oviposition process and to identify active constituents.

METHODS AND MATERIALS

Cultivars, Race Stocks, and Agronomic Practices. Day-neutral BC_2F_4 lines of T-326 and T-1180; a commercial cultivar, Stoneville 213 (ST213); and a breeding line, Stoneville 213 glandless (ST213 gl), were grown in field plots to produce flower buds for the various chemical analyses. These four plus nine other lines were analyzed for bud surface chemicals. The day-neutral lines, T-326 DN and T-1180 DN, hereafter referred to without DN, previously were reported to carry resistance to boll weevil oviposition (McCarty et al., 1987). Stoneville 213 and Stoneville 213 glandless served as susceptible controls. Standard cultural practices were followed during the growing season. All plant material was collected from cotton plants of similar maturity.

Laboratory Bioassays, Coated Buds, Oviposition Plugs, and Handling of

Insects. Boll weevils used in the bioassays were obtained from a small colony maintained on standard artificial diet at the Boll Weevil Research Laboratory. Wild males are introduced into this colony yearly to maintain the genetic diversity of the colony. Boll weevils were removed from the colony five to seven days after emergence, and sex was determined.

Coated-Bud Bioassay. Freshly collected cotton buds (8 mm diameter) from ST213 gl with bracts removed were coated with 250 μl ethyl ether or ethanol containing 250 μg of test material. The coated bud was placed in a plastic cage (2.54 cm^3) with one female weevil. The female was allowed to feed and oviposit on the test bud, in a no-choice situation, for 4 hr after which it was removed. The bud was then examined for feeding and oviposition punctures using a binocular microscope, dissected, and the number of eggs was recorded (a feeding puncture is an open puncture, while an oviposition puncture is sealed and contains an egg). The tests were run at a temperature of 29 $\pm$ 1°C, a relative humidity of 70 $\pm$ 5%, and under normal laboratory light conditions.

Oviposition Bioassay. The test chemical or isolate was incorporated in a 2% agar cylindrical plug (8 mm diameter, 12 mm length = 0.603 cc^3) that was coated with a mixture of 40% beeswax and 60% paraffin (see tables, below, for concentrations). Twenty diet plugs were produced for each test chemical. Each agar plug was placed in a plastic cage (2.54 cm^3) with one female boll weevil. The female was allowed to feed and oviposit for 4 hr. The agar plug was then examined using a binocular microscope and the number of eggs was recorded. A control consisting of a 2% agar diet plug was used with each bioassay. The laboratory conditions were the same as for the coated bud bioassay.

Chemical Analyses of Flower Bud Nutrients. Association of Official Analytical Chemists (AOAC) methods (Horwitz, 1975) were used for the following analyses: total solids (moisture), 14.083; crude fat, 14.019; crude fiber, 14.118; ash, 14.114; total protein, 2.049 (percent nitrogen $\times$ 6.25); nitrogen-free extract (NFE), by difference from 100%. Total sugars were determined by the colorimetric anthrone procedure. Protein was also determined colorimetrically by the Coomassie blue test.

Analysis of Allelochemicals. Analyses for gossypol and related terpenoid aldehydes were performed on cyclohexane–ethyl acetate–acetic acid (500:500:1; CHEA) extracts of plant (whole bud and anther) tissue by the phloroglucinol reaction [2% in absolute EtOH–concentrated HCl (1:1); stand 1 hr] with subsequent spectrometric analysis at 550 nm. The concentration was determined by comparison with data obtained from authentic gossypol and is expressed as gossypol equivalents. Condensed tannin analyses were performed on 70% aqueous methanol (MW) extracts of tissue. The anthocyanidin chromophore was developed from the tannin by boiling 1 hr with 1-butanol–HCl (95:5) (Hedin et al., 1982). The concentration was determined by comparison with the color obtained at 550 nm from a purified cotton condensed tannin sam-

ple, the structure of which was elucidated by Collum et al. (1981). The anthocyanin content was determined by measuring the absorbancy at 540 nm of an extract of freeze-dried tissue extracted with methanol–water–HCl (79:19:3), using the molar extinction coefficient (E) of cyanidin 3-β-glucoside (Hedin et al., 1967). Flavonoids were determined after extraction of freeze-dehydrated tissue with 70% aqueous acetone. Diphenylboric acid–ethanolamine complex (Natural Product Reagent A. Aldrich Chemical Co., 1%) in methanol was added, and the chromophore absorptivity at 440 nm was determined and compared to that obtained from a purified sample of isoquercitrin, the most prevalent flavonoid in cotton.

Isolation of Wax Esters. Anthers (3 g) were ground in chloroform–methanol–water (2:1:1). The nonpolar phase was concentrated and chromatographed on a 2 × 18-cm Biosil A column (Biorad Laboratories, Richmond, California) with chloroform, which eluted the wax esters as a yellow band. CI-MS (methane) via solid probe revealed the presence of a mixture of wax esters based on comparison of the fragmentation patterns with those of a number of synthetic esters. IR provided additional confirmation for the presence of the esters.

Synthetic Wax Esters. The esters were prepared via reaction of the acid with an excess of thionyl chloride in benzene at reflux temperature according to the procedure of McKibben et al. (1985). After removal of solvent and excess thionyl chloride, the acid chloride was redissolved in dry benzene and added to a molar equivalent of the alcohol and pyridine in dry benzene. The reaction mixture was heated overnight at 60°C, then solvent was removed under vacuum and the dry residue was triturated with hexane. The hexane-soluble material was chromatographed over Biosil A with the esters generally being eluted with methylene chloride. The fractions were analyzed by TLC and synthesis was confirmed by CI-MS. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri) except geranyl-geraniol, which was a gift from Dr. Malcolm Thompson, USDA, Beltsville, Maryland. In some cases, the ester or the acid chloride were commercially available from Sigma, obviating part or all of the synthetic work.

Isolation of Anther Water-Soluble Protein, Amino Acids, and Sugars. Water extracts of homogenized anthers (5 g in 100 ml H_2O, repeat and combine) were freeze-dried and then redissolved in water and chromatographed on a 5 × 70-cm Sephadex G-50-150 column with water as the eluant. Eight to ten 250-ml fractions were collected with the proteins eluting in the first 750 ml, and the amino acids and sugars eluting together from 1200 to 1600 ml. The amino acid–sugar fraction was freeze-dried and reconstituted in water prior to chromatography on a 2 × 6-cm Dowex 50 column that was prepared by equilibration with HCl followed by washing with water. When the sample was applied to the column, the sugars were eluted with water until reaction of aliquots with

anthrone demonstrated no further presence of sugars. The amino acids were subsequently eluted with 10% NH_4OH until reaction of aliquots with ninhydrin demonstrated that all of the amino acids had been eluted. The sample fractions were again freeze-dried prior to analysis.

GLC-MS Analysis of Cotton Bud Surface Chemicals. Approximately 30 g of cotton buds (13 lines for this only) were stirred in 200 ml of ethyl ether for 5 min. The ether was decanted, and the process was repeated with a second 200-ml portion of ethyl ether. The combined washings were concentrated to 10 ml under reduced pressure. Then 0.5–1.0 μl was analyzed by GLC–EI–MS on a DB-1 fused silica column (15 m $\times$ 0.322 mm) that was interfaced to a Hewlett Packard 5985-B quadrupole mass spectrometer through an open-split interface. The GLC was programmed from 70 to 250°C at 10°/min. An approximation of concentrations of components was obtained by comparing the MS data system total abundance count of the ion chromatograms with that of standards.

HPLC Analyses of Terpenoid Aldehydes, Amino Acids, and Sugars. HPLC analysis of the gossypol-type terpenoid aldehydes was performed on a reverse-phase C_{18} column according to the procedure of Stipanovic et al. (1988), who also kindly provided samples of hemigossypolone, and heliocides H_1 and H_2 for column calibration. Amino acids were determined by HPLC analysis after hydrolysis of anther tissue, employing their phenylthiocarbamyl derivatives (Cohen et al., 1986). Individual sugars were determined by HPLC analysis, employing a Waters μBondapak NH_4 column and acetonitrile–water (80:20).

Procurement of Chemicals. All commercially available chemicals were obtained from Sigma Chemical Co. St. Louis, Missouri. Gossypol was a gift from the Southern Regional Research Center, USDA, New Orleans, Louisiana.

RESULTS AND DISCUSSION

Bud Surface Terpenoids. The terpenoids present on the bud surface were stripped with ethyl ether and subsequently analyzed by GLC-MS. It is shown in Table 1 that β-caryophyllene was the most prevalent terpenoid in all susceptible (S) and resistant (R) lines. The total terpene content was generally higher in R lines than S lines. The glandless line, ST213 gl, possesses almost no terpenes, in confirmation of the report of Elzen et al. (1985) and Chang et al. (1988). After the terpenoids were chromatographed to yield hydrocarbon and alcohol fractions, it was determined that the hydrocarbons had no effect on oviposition. Although not statistically significant, the alcohol fraction appeared to decrease oviposition (Table 2).

Effects of Anthers and Anther Extracts on Oviposition. In the first test (Table 3), isolates from a two-phase extraction carried out with chloroform : methanol : water (2 : 1 : 1) were coated onto bud surfaces of ST213 gl. The

TABLE 1. TERPENOIDS RECOVERED FROM COTTON BUD SURFACE OF WHOLE BUDS; AVERAGE OF TRIPLICATE ANALYSES

Line	Boll weevil reaction[a]	Terpenoids (μg/g) Total C_{10}	β-Caryophyllene	α-Humulene	β-Bisabolol	Total other C_{15}	Total
T-1180	R	19.1	13.6	4.2	4.5	8.3	49.7 ± 5.4
85-9143	R	13.8	17.6	5.0	3.1	2.8	42.3 ± 5.1
T-326	R	11.6	12.9	3.3	0.0	1.8	29.6 ± 3.5
ST213	S	12.2	4.5	1.2	2.9	5.4	26.2 ± 9.8
COKER-310	S	10.4	7.5	1.9	0.0	1.7	21.5 ± 5.6
DPL-41	S	5.9	10.6	2.8	0.0	1.6	20.9 ± 4.3
85-9145	R	7.4	7.4	2.0	0.0	3.0	19.8 ± 4.0
ST825	S	4.1	4.7	1.3	3.5	4.5	18.1 ± 5.3
CAMD-E	S	5.3	5.0	1.3	1.9	2.8	16.3 ± 2.0
DELCOTT	S	3.0	4.7	1.3	1.5	2.5	13.0 ± 4.1
ACALA-SJ-5	S	2.4	3.1	0.9	1.0	4.1	11.5 ± 2.6
DPL-61	S	1.4	5.0	1.4	0.0	0.8	8.6 ± 3.4
ST213 gl	S	0.0	0.8	0.0	0.0	0.0	0.8 ± 0.2

[a] R = resistant, S = susceptible.

nonpolar fraction of the R line T-326 significantly decreased oviposition, and T-1180 also appeared to depress oviposition relative to the S lines ST213 and ST213 gl. The oviposition depression caused by the nonpolar phase of ST213 relative to that of ST213 gl probably can be attributed to gossypol in the former, although this may be of limited importance because high gossypol lines do not

TABLE 2. EFFECTS OF COTTON BUD SURFACE CHEMICALS ON BOLL WEEVIL OVIPOSITION[a]

Line	Ovipositions (% of agar control)[a,b] Terpene hydrocarbons	Terpene alcohols
ST213	95a	86a
T-326	95a	76a
T-1180	105a	67a
Control	100a	100a

[a] Buds stripped of surface chemicals by ethyl ether soak, extract chromatographed on silicic acid, and 250 μg applied to bud surface on ST213 gl.

[b] Based on five replications. Means within a column followed by the same letters were not significantly different at the 0.05 level of probability according to a Duncan's multiple-range test.

TABLE 3. EFFECT OF ANTHER EXTRACTS ON BOLL WEEVIL OVIPOSITION[a]

Line	Ovipositions (% of control)[b]	
	Nonpolar	Polar
ST213 gl	155a	100a
ST213	82ab	95a
T-326	27b	82a
T-1180	55ab	45a
Control	100ab	100a

[a] Two-phase extraction from chloroform–methanol–water (2 : 1 : 1) 250 μg applied to bud surface of ST213 gl.
[b] Based on 10 replications. Means within a column followed by the same letters were not significantly different at the 0.10 level of probability according to a Duncan's multiple-range test.

strongly deter boll weevil oviposition in the field (Lambert et al., 1980; McCarty, unpublished data).

Effects of Anther and Synthetic Wax Esters on Oviposition. To determine whether the wax esters in cotton buds reported by McKibben et al. (1985) to the boll weevil feeding stimulants also affected oviposition, the following tests were carried out. Anthers were preextracted with ethyl ether to remove the wax esters. Ground, ether-extracted and nonextracted anthers were incorporated into feeding plugs. It is shown in Table 4 that oviposition punctures appeared to be

TABLE 4. EFFECTS OF FREEZE-DRIED ANTHERS INCORPORATED INTO AGAR PLUGS ON BOLL WEEVIL OVIPOSITION

Line	Amount (mg/agar plug)[a]	Ovipositions (% of agar control)[b]		
		Original	Less waxes[c]	Isolated waxes[d]
ST213	25	85a	90a	105a
ST213 gl	25	90a	85a	100a
T-326	25	60a	50a	115a
T-1180	25	65a	50a	110a
Control	0	100a	100a	100a

[a] 0.5 g freeze-dried anthers added to 0.4 g agar and 20 ml water. Mixture was blended, boiled, and poured into the 8-mm-diameter holes of a 20-hole, 12-mm-thick aluminum plate, allowed to gel on cooling, extruded and dipped into melted beeswax–paraffin mixture to yield 20 agar plugs.
[b] Agar control yielded 20 oviposition punctures each containing eggs. Based on 20 replicates. Means within a column followed by the same letters were not significantly different at the 0.05 level of probability according to a Duncan's Multiple Range Test.
[c] Anthers were extracted with ethyl ether to remove waxes. Residue was tested in bioassay.
[d] Ether extract resuspended in 0.5 ml ethanol and added to agar and water as in [a] above.

lower on the R anther lines, although this was not statistically significant. The same trend, although also not significant, was observed when ether-extracted anthers were bioassayed. All of the isolated waxes were slightly stimulatory.

Fragmentation patterns obtained by solid probe CI-MS, IR, and TLC showed that several wax esters were present in the ethyl ether extract of anthers, particularly in the R lines T-326 and T-1180. ST213 and ST213 gl contained only about 20% of that in T-326 and T-1180. By comparison with the CI-MS obtained for 17 synthetic esters, the presence of oleyl oleate, oleyl phytol, palmityl cholesterol, and palmityl and oleyl sitosterol could be presumptively detected in the ether extracts. These compounds and others had been found previously in cotton anthers by McKibben et al. (1985) and Lusby et al. (1987).

The ovipositional activity of the synthetic wax esters was bioassayed at one or more concentrations using feeding plugs. According to the results shown in Table 5, most appeared to be mildly stimulatory, and three (two of which were sterol esters) appeared to be mildly suppressant, although none of the results were significantly significant. Behenyl geranylgeraniol and lauryl phytol, compounds found by McKibben et al. (1985) to be the most feeding stimulatory of the compounds they tested, tended to stimulate oviposition mildly. The results obtained with these synthetic feeding stimulatory wax esters indicated that their potency did not appear to extend to either inhibition or enhancement of oviposition, hence further purification work on our wax esters isolates did not seem warranted.

Effects of Cotton Allelochemicals on Oviposition. The whole buds and their excised anthers were analyzed for the major allelochemicals in cotton. Gossypol was significantly higher in the anthers of ST213 than in the two R lines (Table 6) and also appeared to be slightly higher in the whole bud. ST213 gl was very low, as expected. The gossypol content of anthers was several-fold higher than in the whole buds within lines. When glands were removed by dissection from the anthers, and the glands extracted with cyclohexane–ethyl acetate–acetic acid (500:500:1) (CHEA), gossypol was found in ST213 glands, but the glands were nearly devoid of gossypol in the two R lines. Previous work by us (Belcher et al., 1983) had shown ST213 anther glands contained 43% gossypol. Bell and Stipanovic (1977) reported even higher gland gossypol (about 80%). Flavonoids appeared to be slightly lower in buds of the two R lines. Tannins and anthocyanins were much higher in buds of all lines than in anthers. The only major difference in allelochemicals between S and R lines was the lower gossypol in anthers of the two R lines.

Because gossypol is known to coexist in the plant with a number of related terpenoid aldehydes, the gossypol fraction was investigated by HPLC. Of the six major terpenoid aldehyde components, four of which were identified, all are present in ST213 and the two R lines, and there are no major differences in their distribution that could likely account for differences in oviposition by the boll weevil on these lines (Table 7).

TABLE 5. EFFECT OF SYNTHETIC WAX ESTERS ON BOLL WEEVIL OVIPOSITION

Acohol moiety	Acid moiety	Concentration (μg/plug)	Ovipositions (% of control)
Palmityl alcohol	C16	125	111
		500	156
	C16	500	122
Oleyl alcohol	C18:1[b]	125	124
		500	108
Phytol	C12	500	111
	C16	125	103
		500	92
	C18:1[b]	125	92
		500	131
	C20	125	117
		500	97
	C22	125	132
		500	126
Geranyl geraniol	C18:1	125	111
		500	100
	C22	250	116
		500	159
Pentadecanol	C16	500	156
	C18:1	500	78
Cholesterol	C16[b]	125	126
		500	70
	C18:1	125	108
		500	135
Sitosterol	C16[b]	125	76
		500	123
	C18:1[b]	125	133
		500	126

[a] Differences from the control were not significant according to a least significant difference test at the 0.05 level.
[b] Found in wax ester isolate from T-326 and T-1180.

Finally, gossypol and several other cotton constituents were tested for their effects on oviposition in the feeding plug bioassay (Table 8). The levels tested were those present in anthers. Gossypol, a known feeding stimulant (Hedin et al., 1968), appeared to be an oviposition depressant, although not significantly so, but as previously mentioned, moderately high gossypol lines are not resistant to the boll weevil (Lambert et al., 1980; McCarty, unpublished data). Scopoletin, reported to be present in cotton plants by Wakelyn et al. (1984), was significantly depressant at the higher level. Scopoletin was not found by us in these lines, although we did obtain MS evidence for a trace of scoparone, which

TABLE 6. ALLELOCHEMICALS OF BOLL WEEVIL OVIPOSITION SUPPRESSANT LINES IN BUDS AND ANTHERS[a]

Line	Gossypol	Flavonoids	Tannins	Anthocyanin
Buds (%)				
ST213 gl	0.08 ± 0.01	0.47 ± 0.03	12.86 ± 0.76	0.10 ± 0.01
ST213	0.28 ± 0.04	0.48 ± 0.07	13.49 ± 2.30	0.13 ± 0.01
T-326[b]	0.23 ± 0.04	0.41 ± 0.08	10.08 ± 4.25	0.15 ± 0.03
T-1180[b]	0.26 ± 0.05	0.33 ± 0.02	13.58 ± 0.88	0.15 ± 0.01
Anthers (%)				
ST213 gl	0.21 ± 0.02	0.47 ± 0.04	2.58 ± 0.52	0.02 ± 0.01
ST213	1.00 ± 0.06	0.49 ± 0.06	2.48 ± 0.62	0.02 ± 0.00
T-326[b]	0.77 ± 0.07	0.47 ± 0.03	2.07 ± 0.15	0.02 ± 0.01
T-1180[b]	0.79 ± 0.05	0.46 ± 0.06	3.75 ± 0.32	0.02 ± 0.02

[a] Average of three analyses.
[b] T-326, T-1180.

had been found in cotton by Chan and Wilson (1988), and appeared to be mildly stimulatory (Table 8).

Palmitic acid appeared to be a mild oviposition stimulant while methyl linolenate was not active. Methyl linolenate was identified as an important feeding deterrent for the boll weevil in rose-of-Sharon, *Hibiscus syriacus* (L.), calyx, while palmitic acid, also present in *H. syriacus* calyx, was stimulatory (Bird et al., 1987).

The most active boll weevil plant attractant, β-bisabolol (Minyard et al.,

TABLE 7. HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF COTTON TERPENOID ALDEHYDES

Compound	Percent of total content			
	ST213	T-326	T-1180	ST213 gl
Hemigossypolone	8.6	8.2	10.7	ND[d]
M^+514[a]	23.0	21.4	20.2	ND
10.70[b]	4.7	5.5	4.5	ND
Gossypol	33.3	27.0	22.7	ND
Heliocide H_1	11.9	16.4	17.8	ND
Heliocide H_2	18.5	21.8	24.1	ND
Total Gossypol, Percent[c]	0.28 ± 0.04	0.23 ± 0.04	0.26 ± 0.03	0.08 ± 0.02

[a] HPLC maxima giving a molecular ion (MS) of M^+514.
[b] HPLC maxima with retention time of 10.70 min.
[c] Total gossypol as determined by phloroglucinol procedure.
[d] Not detectable.

TABLE 8. EFFECTS OF KNOWN COTTON CONSTITUENTS INCORPORATED IN AGAR PLUGS[a] ON BOLL WEEVIL OVIPOSITION

Compound	Concentration (mg/diet plug)	Ovipositions (% of agar control)
Gossypol	0.25	32
	0.50	78
Scopoletin	0.50	55
	3.00	21[b]
Scoparone	0.50	127
Palmitic acid	0.50	122
Methyl linolenate	0.50	94
β-Bisabolol	0.08	90
	0.25	36
β-Caryophyllene	0.25	98

[a] Constituents dissolved in 0.2–0.5 ml ethanol and added to 0.4 g agar and 20 ml water. The mixture was boiled, poured into the 8-mm-diameter holes of a 20-hole, 12-mm-thick aluminum plate, dipped into a melted beeswax–paraffin mixture to yield 20 agar plugs.
[b] Significantly different from the control according to a least significant difference test at the 0.05 level.

1969) appeared to be oviposition depressant at the 0.25-mg level (although not statistically so) while β-caryophyllene, also found to be attractive to the boll weevil, was not active. Overall, gossypol and β-bisabolol may contribute to suppression of oviposition, but by themselves do not appear to account for the total activity if consideration is given to the results of Tables 3 and 4, which indicate other classes of compounds also contribute to suppression of oviposition.

Effects of Anther Amino Acids, Protein, and Sugars on Oviposition. When anthers were analyzed for these nutrients (Table 9), protein was not different in the S and R lines, but the free amino acids were lower in ST213 and ST213 gl than in the two R lines. Nitrogen-free-extract (essentially total carbohydrates)

TABLE 9. PROTEIN, FREE AMINO ACIDS, TOTAL CARBOHYDRATES, AND FREE SUGAR CONTENT OF COTTON ANTHERS (AVERAGE OF THREE TESTS)

Variety	Protein (%)	Amino acids (%)	Nitrogen-free extract (%)	Free sugars (%)
ST213 gl	27.8 ± 0.8	5.3 ± 0.4	45.3 ± 1.3	6.8 ± 0.7
ST213	27.5 ± 1.1	3.3 ± 0.5	45.2 ± 2.2	12.3 ± 1.3
T-326	27.2 ± 0.4	5.0 ± 0.4	45.6 ± 2.9	9.4 ± 2.4
T-1180	26.6 ± 1.3	6.9 ± 0.4	46.9 ± 2.1	6.5 ± 1.1

TABLE 10. FREE SUGARS RECOVERED FROM COTTON ANTHER TOTAL WATER-EXTRACTABLE SOLIDS BY HPLC (AVERAGE OF THREE TESTS)

Variety	Total water extractable solids (%)	Free sugars recovered by HPLC analysis (%)				
		Fructose	Glucose	Sucrose	Maltose	Others
ST213 gl	46.8 ± 4.0	19.2 ± 0.7	10.8 ± 1.2	2.1 ± 0.1	8.3 ± 1.2	15.6 ± 1.3
ST213	41.4 ± 1.2	16.2 ± 0.8	10.2 ± 0.5	1.8 ± 0.2	9.0 ± 1.1	14.6 ± 1.5
T-326	39.9 ± 1.7	14.7 ± 2.4	10.8 ± 1.0	1.7 ± 0.2	8.0 ± 1.9	14.0 ± 1.2
T-1180	45.6 ± 1.7	10.8 ± 2.0	9.6 ± 1.4	1.6 ± 0.6	5.3 ± 1.0	15.5 ± 2.4

was not different in the S and R lines, but free sugars were at least 30% higher in ST213 than in the two R lines and the glandless line. In a second test, the free sugar content of ST213 was again higher than those of T-326 and T-1180.

The results of HPLC analysis of the total water-extractable solids of anthers are presented in Table 10. Comparable yields (39.9–46.8%) of solids were extractable from the four lines. The sugars fructose, glucose, sucrose, and maltose were recovered from each of the lines. Other maxima were present for which assignments could not be made, 42–65% of the solids being accounted for by these sugars. Notably, fructose was lower in the two R lines.

Free amino acid and protein isolates from anthers and, for comparison,

TABLE 11. EFFECT OF COTTON ANTHER FREE AMINO ACIDS AND PROTEIN INCORPORATED INTO DIET PLUGS ON BOLL WEEVIL OVIPOSITION

Sample	Concentration, (mg/agar plug)	Ovipositions[a] (% of agar control)	
		Amino acids	Protein
Glycine	1	91	
Casein amio acids[b]	3	111	
	10	142	
ST213	10	100	103
	100	167	127
ST213 gl	10	140	73
	100	140	93
T-326	10	87	77
	100	133	93
T-1180	10	133	116
	100	97	63

[a] Oviposition punctures containing eggs. Differences from the control were not significant according to a Least Significant Difference test at the 0.05 level.

[b] Mixture formulated on the basis of the analyzed amino acid content in casein. Percent of total: Asp 6.4, Glu 20.0, Ser 5.7, Gly 2.5, His 2.8, Arg 3.7, Thr 4.4, Ala 2.7, Pro 10.1, Tyr 5.7, Val 6.5, Met 2.5, Cys 0.3, Ileu 5.4, Leu 8.2, Phe 4.5, Lys 7.3, Try 1.5.

glycine and an amino acid mixture, were incorporated into feeding plugs for boll weevil oviposition tests (Table 11). Although none of the results was statistically significant, protein isolates of the two R lines and ST213 gl appeared to mildly depress oviposition, while the amino acid isolates tended to stimulate (up to 67%) oviposition. Glycine alone did not appear to have an effect, but a mixture of amino acids based on analysis for casein appeared to be mildly stimulatory.

Sugar isolates from anthers and several commercially procured sugars were likewise incorporated into feeding plugs at levels of 1.7–16.7% (compare with free sugars in anthers: 6.5–12.3%, Table 9) for boll weevil oviposition tests (Table 12). The sugar isolates from all lines, although not statistically significant, appeared to stimulate oviposition. The authentic sugars were all moder-

TABLE 12. EFFECTS OF COTTON ANTHER SUGAR ISOLATES AND STANDARD SUGARS INCORPORATED INTO DIET PLUGS ON BOLL WEEVIL OVIPOSITION

Sample	Concentration, (mg/agar plugs)[a]	Ovipositions (% of agar control)
D-Glucose	3	120
	10	200[b]
	100	244[b]
D-Fructose	3	131
	10	120
	100	290[b]
Sucrose	0.5	245[b]
	3	191
	20	258[b]
	100	222[b]
Maltose	20	118
	100	188
Lactose	20	231[b]
	100	177
Myo-inositol	3	63
	10	116
	100	136
ST213	10	151
	100	165
ST213 gl	10	142
	100	131
T-326	10	168
	100	162
T-1180	10	176
	100	124

[a] 10 mg/0.6 cc^3 (ml) plug = 1.7%, 100 mg/0.6 cc^3 plug = 16.7%. Compare with free sugars in anthers (6.5–12.3%, Table 9).

[b] Significantly different from the control according to a Least Significant Difference test at the 0.05 level.

ately to strongly stimulatory except for inositol, giving responses of 19–145% above that of the control at levels equal to or below those in anthers (Table 12).

SUMMARY

Laboratory evidence was obtained that some factors present in cotton lines resistant to the boll weevil may contribute to deterrence of oviposition. The surface chemicals of cotton buds were isolated and analyzed by GLC-MS. The R lines were found to possess a higher concentration of volatile terpenoids, especially the sesquiterpenes, than the S lines. However, bioassays did not show that the terpenoid extracts, or standard terpenoids, suppressed oviposition. Nevertheless, there may be a correlation between surface terpenoids and oviposition resistance, and perhaps, some other bioassay could demonstrate a causative basis.

The wax esters may be important boll weevil feeding stimulants, but we were not able to demonstrate that they possessed any substantial effect on oviposition.

Total gossypol was higher in anthers of the susceptible ST213 line than in the two R lines. Paradoxically, gossypol appeared to depress oviposition in laboratory tests even though it previously was shown in other laboratory tests to be a feeding stimulant (Hedin et al., 1968). Because high gossypol lines have not been shown to be boll weevil resistant, the role of gossypol in oviposition does not appear to have been established.

Free sugars stimulated oviposition more consistently and to a greater degree than any other class of constituents. The free sugar content in the R lines was lower than in ST213. If this trend can be confirmed with a number of R lines, it may be established that a major basis of resistance to boll weevils (as related to oviposition) is the decreased content of free sugars in the R lines and, therefore, nutritional. Carbohydrate metabolism in these lines may favor conversion of sugars to bound carbohydrates. There is precedence for the importance of sugars in the report of Dean and Chapman (1973) which indicates that percent sucrose and total sugars contributed to the oviposition of apple maggot, *Rhagoletis pomenella* (Walsh).

The significance of these findings, if further supported, is that the analysis of free sugars in the anther, and perhaps also the analyses of bud surface terpenoids, may provide a basis for selection or genetic production of cotton lines resistant to the boll weevil.

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MALE EUROPEAN CORN BORER, *Ostrinia nubilalis* (HÜBNER), ANTENNAL RESPONSES TO ANALOGS OF ITS SEX PHEROMONE

Strain, Electroantennogram, and Behavior Relationships

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Abstract—Experiments were conducted to (1) determine whether the electroantennogram (EAG) can detect differences among the responses of antennae from males derived from the three strains of *Ostrinia nubilalis* (Hübner), and (2) characterize the EAG responses of each strain to isomeric forms of the natural pheromone, (*E*)- and (*Z*)-11-tetradecen-1-ol acetate (TDA), and analogs possessing differences in the terminal alkyl group, cyclopropyl (CPA), or *tert*-butyl (TBA).

EAG responses differed among the strains in two ways: (1) Antennae from *ZZ* males always produced an EAG to (*Z*)-TDA with an extended duration of response. This "signature" EAG response was found to be unique to the antennal response of *ZZ* males to (*Z*)-TDA, thus providing a relatively easy method of distinguishing live *ZZ* males from *EE* or *ZE* males. Correlated with this longer EAG response was a longer disadaptation time, i.e., the EAG response of *ZZ* antennae disadapted more slowly (ca. 10 min) than the response of *EE* antennae. (2) Strain differences in the relative EAG amplitudes to isomers and analogs were observed at the stimulus amounts eliciting the peak EAG amplitude as follows: TDA $\geq$ CPA $>$ TBA for *ZZ* males and both isomers; TDA $>$ CPA $\geq$ TBA and CPA $\geq$ TDA $>$ TBA for *EE* males and the *E* and *Z* isomers, respectively; CPA $>$ TBA $\geq$ TDA for *ZE* males and both isomers. Dose–response relationships were seen for all compounds if amplitude ("peak height") of the EAG was used as a measure of response. However, if width of the EAG at half the peak height ("peak width") was used, then only the *ZZ* antennal response to (*Z*)-TDA resulted in a meaningful

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dose–response relationship. For all strains, the EAG amplitudes elicited by the *Z* isomers of any of the tested compounds were greater than those elicited by the corresponding *E* isomers. Therefore, correlations between the relative EAG and upwind flight responses were observed in the *ZZ* ($r = 0.86$) and *ZE* ($r = 0.80$) strains but were not correlated in the *EE* strain ($r = 0.18$). Temporal studies showed that adaptation, not postexcision deterioration, was responsible for the observed decreases in the EAG amplitude after repetitive stimulation or after stimulation with amounts in a descending order. Disadaptation required at least 20 min for a moderate dose (10 μg for 1 sec). Developmental studies showed that antennae from 2-day-old adults had the greatest EAG response.

Key Words—Adaptation, chemoreception, electroantennogram, European corn borer, Lepidoptera, *Ostrinia nubilalis*, Pyralidae, sex pheromone, genetic strains, 11-tetradecen-1-ol acetate.

INTRODUCTION

Sexual behavior in the adult European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), is elicited by two geometric isomers of 11-tetradecen-1-ol acetate (TDA) (Klun et al., 1973; Kochansky et al., 1975). Three genetic strains (*ZZ*, *EE*, and *ZE*) of *O. nubilalis* adults can be distinguished by the ratios of (*E*)- and (*Z*)-TDA emitted by the female (Klun and Maini, 1979). Males respond preferentially to the pheromone blend emitted by females of their own strain (Klun and Maini, 1979). Differences between the electroantennogram (EAG) responses of the three strains of *O. nubilalis* males were observed by Nagai et al. (1977). Schwarz et al. (1989) observed strain differences in the behavioral responses of *O. nubilalis* males to analogs of TDA, which they hypothesized to be due to strain differences in the structure of the antennal receptors.

Therefore, the following study was undertaken to determine if the EAG technique will detect strain differences in the male *O. nubilalis* antennal response, not only to the natural pheromone as shown by Nagai et al. (1977), but also to the pheromone analogs tested by Schwarz et al. (1989). The EAG as an empirical tool has correlated well with the behavioral responses of many insects (Kaissling, 1971; Roelofs, 1977). For example, male pheromone morphs of the larch bud moth, *Zeiraphera diniana* (Guenée), could be distinguished by their EAG responses to the pheromone composition of sibling females (Priesner, 1979).

The EAG is hypothesized to be the summation of receptor potentials from all the excited and inhibited sensory receptor neurons in the antenna (Schneider, 1962; Kaissling, 1971; Mayer et al., 1984). Although it is the action potential(s) that encodes information about the chemical stimulus (Kaissling, 1971;

Kaissling and Thorson, 1980), the EAG is a convenient indicator of the summed neural activity being sent to the brain (Mayer et al., 1984). Transduction of an olfactory stimulus into neural activity requires several molecular steps with different chemical specificities (Kaissling, 1976). Since the EAG provides a measure of the overall transduction process occurring throughout the antenna, this technique seems to be an appropriate way to investigate the ability of male *O. nubilalis* olfactory receptors to distinguish the natural pheromone from pheromone analogs.

The three most important parts of a pheromone molecule necessary for specific interaction with receptors on olfactory neurons are the double bond, polar functional group, and terminal alkyl group (Bestmann and Vostrowsky, 1982; Liljefors et al., 1984, 1987). EAGs have been useful in determining the position and configuration of unsaturation and the functional groups to which the antennae of many moth species are the most sensitive (Roelofs, 1977). Responses observed in both EAG (Nagai et al., 1977) and single sensillum studies (Nagai, 1983; Hansson et al., 1987) to (*E*)- or (*Z*)-TDA indicate that the antennae of male *O. nubilalis* can perceive differences in the configuration of unsaturation about carbons 12 and 13. Behavioral studies of *O. nubilalis* corroborate the findings of the EAG studies in demonstrating the importance of the position of unsaturation (Klun et al., 1973, 1979) and the acetate functional group (Klun and Robinson, 1972). However, the sensitivity of the *O. nubilalis* antenna to changes in the terminal alkyl group has not been determined.

Behavioral (Chapman et al., 1978a,b) and EAG (Bestmann et al., 1980) responses of male *O. nubilalis* to chiral analogs of TDA suggested that the receptor for TDA is chiral and that the manner in which the last four carbons of TDA coil within the receptor is important for binding. Bestmann et al. (1980) also found that EAG responses were greater for open-chain (pentadecenyl) analogs than ring structures. They speculated that this is related to the greater flexibility of the open-chain analogs and the ability of the acceptor to adapt to a less rigid structure. Schwarz et al. (1989) found that only acetate analogs with 13 or 14 carbon chain lengths, in which branching at carbon 13 was limited to one methyl group or a cyclopropyl group, elicited upwind flight from *O. nubilalis* males in a flight tunnel. They also observed that upwind flight responses elicited by isomers of the cyclopropyl analogs vary with strain. This was interpreted by Schwarz et al. (1989) to indicate intrinsic receptor differences in the three pheromonal strains.

Although the above behavioral data suggest that differences exist in receptor specificity among strains, they are not conclusive. It is difficult to determine why certain analogs do not elicit behavior: Is this because the antenna (1) does not detect the compound, or (2) detects the compound, but this detection does not result in a behavioral response? As a first step in attempting to answer this question, the EAG was used to determine the ability of the male *O. nubilalis*

antenna to detect (respond to) analogs of (*E*)- or (*Z*)-TDA in which structural modifications were made at carbon 13. As a second step, we compared relationships between chemical structure and antennal response for the three pheromonal strains, and correlated these with the behavioral results of Schwarz et al. (1989). During the course of this investigation, we determined how EAG responses were affected by adaptation, length of time after antennnal excision, and adult age.

METHODS AND MATERIALS

Insects. O. nubilalis males were obtained from cultures homozygous for *ZZ* or *EE* female sex pheromone production genes, or hybrids (*ZE*) obtained by crossing the *ZZ* and *EE* genotypes (Klun and Huetell, 1988). Designation of male strain was based on the ratio of (*E*)- and (*Z*)-tetradecen-1-ol acetate emitted by sibling females (*ZZ* = 3:97; *EE* = 97:3; *ZE* = 66:35; *E*:*Z*). Cultures were maintained at the Insect Chemical Ecology Laboratory, USDA, Beltsville, Maryland. Moths were reared (Reed et al., 1972) at 80% relative humidity and 16:8 light–dark and 26:19°C temperature regime (lights off at 0800 hr). Water was provided for adults emerging from the pupae. Two-day-old males in the hours 2–6 of scotophase were used in all experiments except for those testing for the effect of adult age. Unmated males were used in all experiments. Synchronously developing adults were obtained by collecting males at the end of each scotophase (ca. 87% of the adults emerge during the scotophase). Preexposure of males to pheromone was prevented by removing newly emerged females from the incubator daily.

Recordings. EAGs were obtained from excised antennal flagella using glass capillary electrodes (ca. 100 μm openings) filled with hemolymph Ringers (Kaissling and Thorson, 1980) and held in metal holders possessing Ag–AgCl bridges in contact with the Ringers solution. To facilitate antennal excision, the insects were immobilized with CO_2 for 30 sec. (No differences in the EAG response were observed among antennae obtained from insects immobolized at 5°C or with CO_2.) The antennal flagellum was cut with microscissors and placed in contact with the reference electrode; after removal of the distal 1–2 segments, the distal (cut) end of the antenna was placed in contact with the active electrode. Surface tension and hydrophilic attraction between the antennal tissue and the Ringers solution at the tip of the electrodes maintained electrical contact and supported the antenna. The ventral scale-free side was positioned horizontally to face a constant air flow delivered from the stimulus jet. Not more than 5 min was required to mount an antenna on the electrodes. Usually the baseline stabilized within the next 5 min; preparations that continued to "drift" were discarded.

Short coaxial cables connected the electrode holders to a chopper-stabilized DC amplifier with a gain of 150 (George Johnson Electronics, Baltimore, Maryland). Built into the amplifier was a 1-mV calibration pulse applied via the reference side of the amplifier and a circuit which rezeroed the baseline at the touch of a button. The EAG signal was displayed and quantified by a recorder–integrator (Hewlett Packard 3392A). The integrator determined the peak height and area and width at half the peak height. The height of the calibration signal was used to determine the EAG peak amplitude in mV.

Stimulation. Compounds tested in this study were geometrical isomers of 11-tetradecen-1-ol acetate (TDA), 13,13-dimethyl-11-tetradecen-1-ol acetate (TBA), and 12-cyclopropyl-11-dodecen-1-ol acetate (CPA) (Schwarz et al., 1986, 1989). Capillary gas chromatographic analysis (Carbowax 20 M and DB-1) showed the compounds to be ca. 98% pure with no cross-contamination between isomers.

We assumed that the stimulus release rate, concentration, and rate of adsorption onto the antennal receptor sites were proportional to the amount of compound placed on a strip of filter paper (4 × 0.8 cm; Whatman No. 1). Since TDA is 1.8× and 2× more volatile than TBA and CPA, respectively, correspondingly greater amounts of TBA and CPA were used. For each cohort of five insects tested, new sets of papers containing stimulus were prepared and stored in sealed amber vials at 4°C between experiments. Only one antenna was tested per individual. The duration of stimulation (1 sec), air flow rates, physical factors (filter paper, cartridge and jet sizes and positions, etc.) and protocol for replicates were constant in all experiments.

The odor delivery system was a modification of a system designed by R. W. Mankin and A. J. Grant (personal communication) and figured in Frazier and Hanson (1986). Compressed breathing air, filtered through ×13 molecular sieve, was split into a dry stimulus air flow (150 ml/min) and a humidified (ca. 80% relative humidity at the antenna) carrier air flow (2000 ml/min), which passed continuously through a glass mixing chamber and then over the antennal preparation. Papers containing stimulus were placed in glass cartridges inserted in the stimulus air flow between Teflon stoppers. The air flowed continuously through the cartridge, resulting in a constant release of stimulus from the paper, but a vacuum air flow (160 ml/min) normally prevented this stimulus from mixing in the glass chamber with air flowing over the antenna. To stimulate the antenna, this vacuum line was closed for 1 sec, thereby diverting the stimulus air flow into the mixing chamber to combine with the carrier air flow. The oval-shaped mixing chamber outlet (2 × 0.5 cm) was positioned 2 mm from the antennal preparation. An exhaust line (10 cm diameter), positioned 5 cm behind the antenna, evacuated air flowing over the antenna to a fume hood.

Experimental Design and Methods for Evaluation of Results. Four types of experiments were performed: (1) The responses and antennae from *ZZ*, *ZE*,

and *EE* males to isomers of TDA, TBA, and CPA were compared by exposing antennae of each strain to ascending doses of each compound. (2) Adaptation was studied by sequentially presenting descending doses of (Z)-TDA to *ZZ* male antennae. (3) Recovery (disadaptation) of the EAG after stimulation of individual antennae with 10 μg of (Z)-TDA was examined in *EE* or *ZZ* males by varying the time between stimulus presentation (intertrial interval) from 5 to 60 min. Each antenna was stimulated only three times and each stimulation was separated by a given interval, i.e., only one interval was tested per antenna. (4) The effect of adult age on the EAG response was examined by stimulating antennae from *ZZ* males of various ages with 10 or 40 μg of (Z)-TDA.

Application of a control stimulus also resulted in an EAG response. This EAG appeared to be produced by the stimulation of antennal mechanoreceptors that are sensitive to the small changes in air flow that occur when the stimulus air flow mixes with the constant air flow. The EAG response to a heptane-treated paper dried for 4 min in a fume hood (control stimulus) was the same as a paper which had no heptane applied to it. In all experiments conducted, the mean control response was calculated from the response elicited by three control stimuli (30 sec between each repetition) that preceded each set of chemical stimuli.

For experiments 1, 2, and 4, delivery of a chemical stimulus to each antenna was replicated three times at 30-sec intervals (amounts 10 μg or lower) or at 1-min intervals (amounts above 10 μg) between each repetition. Each set of three chemical stimuli were followed by 3 min of clean air. The mean response elicited by each set of three replicates was calculated, and the mean control response was subtracted from the mean response to the compound. For each time tested in experiment 3, the mean control response was subtracted from the response to a single delivery of a chemical stimulus.

A factorial design was used to determine the effects of strain, compound, and isomer on the EAG responses calculated for experiment one. Since the treatments were not presented in a random order, the factorial was analyzed by amount of compound. For experiment 2, Student's t test was used to compare mean EAG responses for differences (H_0: $\mu_1 = \mu_2$). A factorial design was used to examine the effects of intertrial interval time and stimulus number (replicate) on disadaptation of the EAG response (experiment 3). A completely randomized design was used to determine the effect of adult age (experiment 4) on the EAG response. Relationships among the EAG amplitude, peak area, and peak width at half the peak height were tested using regression analysis and Pearson product–moment correlations (Steel and Torrie, 1980). The latter were also used to examine the relationship between the EAG and upwind flight activity. Means were separated by Tukey's w procedure (Steel and Torrie, 1980; Jones, 1984). Values in the results section are means plus or minus the standard error. Probability (P) values in the results section are for the independent effects type III sums of squares F test. Student's t test was used to determine if means were

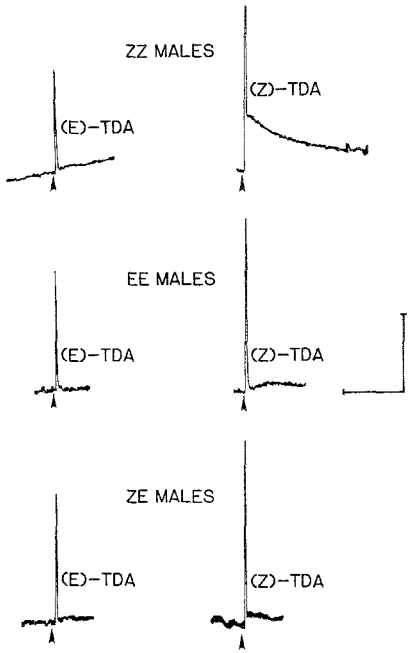

FIG. 1. Typical recordings of the EAGs elicited by 40 μg of (*E*)- or (*Z*)-tetradecen-1-ol acetate (TDA) from the antennae of male *O. nubilalis*. Responses within a strain were obtained from the same preparation. Arrows indicate when the stimulus solenoid was activated. Calibration: 1 mV, 1 min.

different from zero (H_0: $\mu = 0$). SAS was used to perform all statistical analyses and graphs (SAS Institute, 1985, 1987).

RESULTS

Description of EAG. A 1-sec pulse of pheromone elicited an EAG from *O. nubilalis* antennae that was recorded as an initially rapid negative-going change in the background potential to a sharp peak followed by a slightly slower decay phase (Figure 1). The entire response duration was ca. 4–5 sec. The only exception encountered was the response of *ZZ* male antennae to (*Z*)-TDA (10–400 μg). In this case, a slow decay phase was always observed after an initial sharp peak (Figure 1).

The standard measure of the EAG has traditionally been the amplitude (mV) of the peak, which is the primary measure used in this study. Other measures are also plausible: for example, a correlation between amplitude and peak area was observed ($P > |r| < 0.0001$; $r^2 = 0.9$; $N = 2058$). Regression analysis indicated that this correlation was linear [$P < 0.0001$; $r^2 = 0.83$;

N = 2058; peak area = (−9872) + amplitude (678989)], i.e., amplitude and peak area increased together. In contrast, width of the peak at half the peak height was not a good indicator of differences in the EAG. This measure was usually not different from the blank response ($P > |t| > 0.5$). The one exception was the response of *ZZ* male antennae to ascending doses of (*Z*)-TDA, which increased from a width of 0.07 min at 1 μg to a maximum width of 0.97 min at 40 μg and then decreased to a width of 0.55 min at 400 μg.

Experiment 1: Ascending Dose–Response Relationships. As stimuli above 0.1 μg for TDA, 0.2 μg for CPA, and 0.18 μg for TBA were presented to the antennae in ascending order, the EAG amplitudes increased to a peak and then decreased (Figures 2 and 3). Shapes of the ascending dose–response curves for

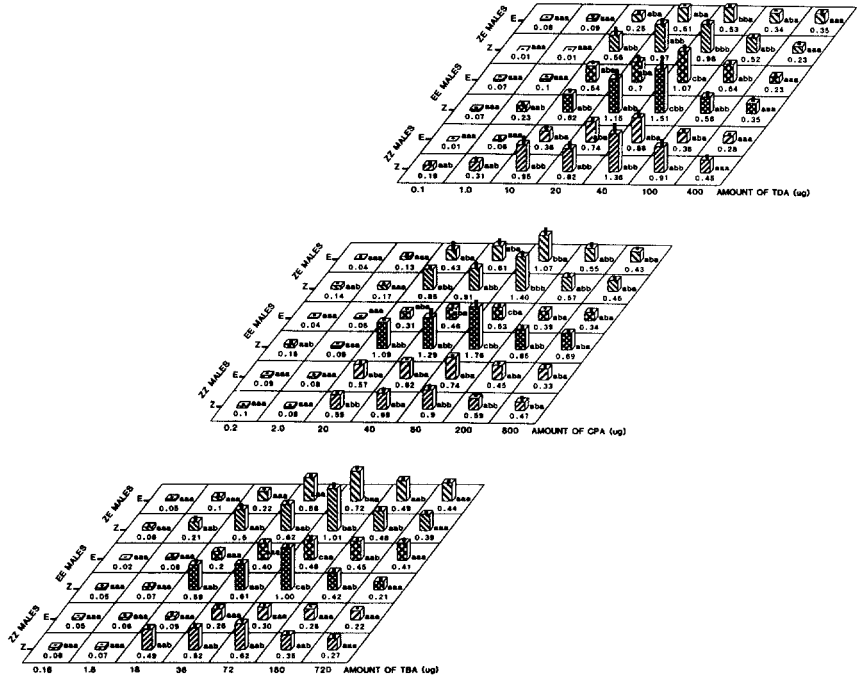

FIG. 2. Ascending dose–response relationships for the amplitude (mV) of the EAGs from male *O. nubilalis* antennae (N = 10–17 per strain, compound, and isomer combination). Vertical bars represent the mean values printed at the base of each bar. The standard error is represented by the smaller solid bar at the top of each mean vertical bar. Means of EAGs elicited by the same amount of compound are not significantly different if followed by the same letter group (α = 0.05; Tukey's w procedure). The first letter in the group denotes significant differences among strains, the second letter denotes significant differences among compounds within a strain, and the third letter denotes significant differences between isomers within a strain and compound.

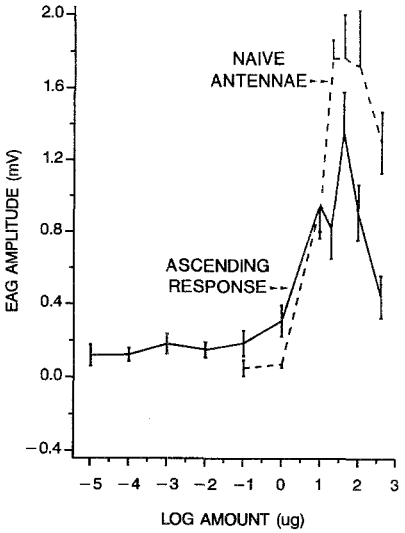

FIG. 3. Comparison of ascending and descending dose–responses for the amplitude of the EAGs from *ZZ* male *O. nubilalis* antennae stimulated with (*Z*)-tetradecen-1-ol acetate. The solid line is the response curve for an ascending series of amounts ($N = 17$). The dashed line is the reconstructed response curve obtained from the first response of the naive antennae used in Table 1 ($N = 10$ for each point). Points represent means ± standard error.

EAG amplitude were similar for each combination of strain, compound, and isomer tested (Figure 2). The maximum amplitude was observed at 40 μg TDA, 80 μg for CPA, and 72 μg for TBA, irrespective of the strain and isomer. EAGs produced by low stimulus amounts (<0.1 μg for TDA, <2.0 μg for CPA, and <0.18 μg for TBA) were not different from zero ($P > |t| > 0.1$).

Interactions among the treatment groups strain, compound and isomer were observed for the EAG amplitudes elicited by doses >1.0 μg ($P < 0.04$). These interactions are reported in Figure 2 and summarized as follows:

1. Responses of the three strains were generally similar. Differences ($P < 0.001$) among strains were only observed at one dose (40 μg of TDA, 80 μg of CPA, and 72 μg of TBA); for example, the *ZE* strain had the lowest response to TDA but usually had the highest responses to CPA and TBA (Figure 2).

2. Differences ($P < 0.03$) in responses among compounds within strains were observed when doses exceeded 1.0 μg of TDA, 2.0 μg of CPA, or 1.8 μg of TBA. Amplitudes of the EAGs elicited by TDA were similar to those elicited by CPA and usually greater than those elicited by TBA (Figure 2).

3. All strains elicited greater responses to the *Z* isomer than to the *E* iso-

mer for doses of all compounds exceeding 1.0 μg ($P < 0.03$), except for the highest doses ($P > 0.15$). The largest differences between EAG responses to isomers were observed when *EE* antennae were stimulated by the isomers of CPA (Figure 2).

Experiment 2: Descending Dose–Response Relationships. Stimulation of antennae using a descending series of amounts of (*Z*)-TDA results in a reduced or eliminated EAG at lower amounts of stimulus (Table 1). This demonstrates that the antenna adapts readily and suggests that even the ascending dose–response curve may be partially depressed at the higher stimulus intensities (>40 μg). This can be shown by reconstructing a dose–response curve (dashed line in Figure 3) using naive antennae for each stimulus dose. At higher doses (>10 μg of TDA), the EAGs in the actual ascending curve are smaller ($P > |t| < 0.005$) than when naive antennae are stimulated with the corresponding amount (i.e., the first value in each line in Table 1). This difference between the two curves in Figure 3 indicates that in the ascending series, the antenna was still partially adapted to the previous stimulation. The largest amount of stimulus that did not result in adaptation was 10 μg of (*Z*)-TDA.

Experiment 3: Disadaptation of EAG Response. The duration of the intertrial interval affected EAG responses of *ZZ* males differently than *EE* males ($P < 0.0047$) (Figure 4). A 15- to 20-min interval between stimulations with 10 μg of (*Z*)-TDA was sufficient to enable antennae from *EE* males to disadapt, whereas *ZZ* males required 20–30 min. The EAG response decreased with increasing stimulus number ($P < 0.048$) or decreasing interval duration ($P < 0.0001$) for intertrial intervals ≤ 15 min (*EE*) or ≤ 20 min (*ZZ*) (Figure 4). When intertrial intervals ≥ 20 (*EE*) min or ≥ 30 min (*ZZ*) were tested, responses to the second and third stimuli were usually greater than responses to the first stimulus ($P < 0.009$) (Figure 4).

These results suggest that antennae disadapt in 15–30 min and suffer no postexcision deterioration with time up to 120 min, which is the total duration of the 60-min intertrial interval experiment (Figure 4). To further distinguish among adaptation and postexcision deterioration, another experiment was conducted in which *ZZ* antennae were excised, mounted on the electrodes, but not stimulated until 105 min after excision; these EAG amplitudes (2.7 ± 0.2 mV) were similar ($P < 0.8$) to the amplitudes (2.61 ± 0.15 mV) recorded at time zero in other experiments (e.g., at stimulus 1 in Figure 4). This similarlity in response shows that the preparations had not deteriorated during the 105-min waiting period between excision and first stimulation.

Experiment 4: Effect of Adult Age on EAG Response. After increasing ($P < 0.0001$) to a peak on the second day after emergence, EAG responses decreased with increasing age to a level that was not different ($P > 0.15$) from 0.5- or 1-day-old adults (Figure 5).

TABLE 1. AMPLITUDE OF EAGS FROM *ZZ* MALE *O. nubilalis* ANTENNAE STIMULATED WITH DESCENDING SERIES OF (*Z*)-TETRADECEN-1-OL ACETATE (TDA) AMOUNTS[a]

Series number	EAG amplitude (mV)[b]							
	400[c]	100[c]	40[c]	20[c]	10[c]	1.00[c]	0.10[c]	0.01[c]
1	1.30 ± 0.17	0.05 ± 0.03	0.02 ± 0.01	0.08 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
2		1.72 ± 0.30	0.25 ± 0.15	0.27 ± 0.10	0.27 ± 0.11	0.04 ± 0.06	0.02 ± 0.05	0.02 ± 0.06
3			1.86 ± 0.24	0.14 ± 0.04	−0.06 ± 0.08	−0.14 ± 0.04	−0.07 ± 0.02	0.05 ± 0.02
4				1.76 ± 0.20	−0.20 ± 0.13	−0.21 ± 0.04	−0.06 ± 0.03	0.01 ± 0.02
5					0.94 ± 0.13	−0.11 ± 0.03	0.05 ± 0.02	0.07 ± 0.03
6						0.07 ± 0.02	0.04 ± 0.03	0.06 ± 0.03
7							0.05 ± 0.04	0.08 ± 0.03

[a] The initial stimulus in a descending series was presented to a naive antenna.
[b] Values are means ± standard error (N = 10 antennae per descending series with a different initial stimulus amount).
[c] Amount of TDA (μg).

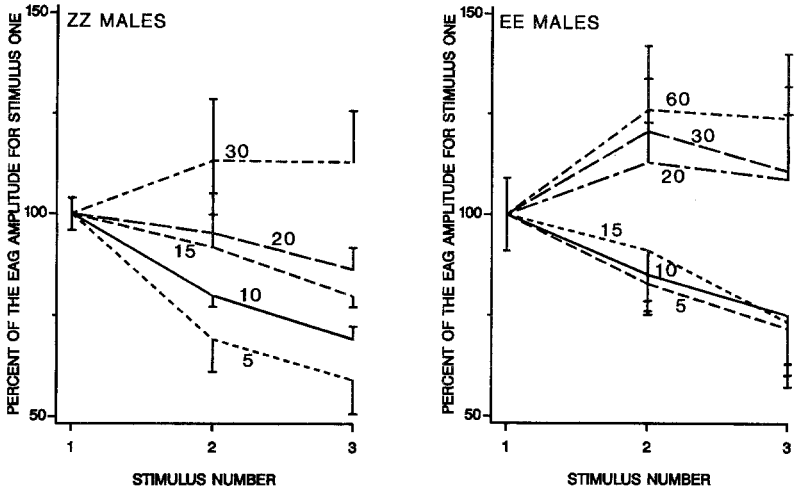

FIG. 4. Disadaptation of the EAG response or the effect of varying intertrial interval time on the amplitude of the EAG response to 10 μg of (*Z*)-tetradecen-1-ol acetate. The intertrial interval (in minutes) is indicated beside each adaptation curve. The mean EAG amplitude to the first stimulus in each series was 2.61 $\pm$ 0.15 and 1.57 $\pm$ 0.12 mV for *ZZ* and *EE* antennae, respectively. Points represent means $\pm$ standard error (N = 8–10 per curve).

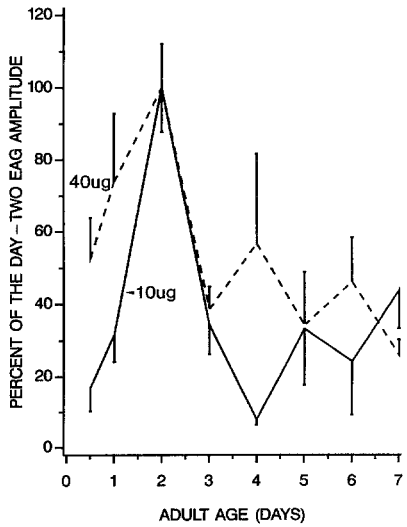

FIG. 5. Effect of adult age on the amplitude of the EAG response. The solid line is the relative EAG response to 10 μg (*Z*)-tetradecen-1-ol acetate and the dashed line is the relative response to 40 μg. Points represent means $\pm$ standard error (N = 5–10 per point).

DISCUSSION

EAG amplitudes elicited by the *Z* isomer were always greater than those elicited by the *E* isomer for all compounds and strains tested. Nagai et al. (1977) and Nagai (1983) also found that (*Z*)-TDA elicited higher EAG and single sensillum DC responses than the *E* isomer in the *ZZ* strain of *O. nubilalis*. However, Nagai et al. (1977) reported that the responses to the two isomers were similar in the *EE* and *ZE* strains, while we found (*Z*)-TDA to elicit a greater EAG response than the *E* isomer in all strains tested. Our EAG results for *O. nubilalis* are consistent with those for noctuid and tortricid species (reviewed in Priesner et al., 1975; Priesner, 1979) that also use straight chain, (*Z*)-monoolefinic acetates as part of the pheromonal complement. In these species, both EAG (Priesner et al., 1975) and single olfactory sensilla (Priesner, 1979) responses from male antennae have shown that the *Z* isomer elicits a significantly greater response than the *E* isomer. This difference in response has been correlated with conformational properties of the entire molecule, i.e., the conformational energy for the *Z* isomer is lower than the *E* isomer (Liljefors et al., 1987). Hansson et al. (1987) showed that for all strains of *O. nubilalis*, a greater single sensillum DC potential is elicited by (*Z*)-TDA than by (*E*)-TDA. This relationship is similar to our data for the EAG amplitude. The results summarized above suggest that, irrespective of strain, the ability of male *O. nubilalis* antennae to detect differences in the stereochemistry of the double bond between carbons 11 and 12 is not affected by the modifications made at carbon 13.

Schwartz et al. (1989) hypothesized that the dissimilarity they observed in behavioral responses of *ZZ* and *EE* males to (*Z*)- and (*E*)-CPA indicated that strain differences existed in the structures of the antennal receptors. In our experiments, strain differences in EAG amplitude were observed, but only at stimulus amounts that elicited the peak EAG response. These differences were: TDA $\geq$ CPA $>$ TBA for *ZZ* males and both isomers; TDA $>$ CPA $\geq$ TBA and CPA $\geq$ TDA $>$ TBA for the *EE* males and *E* and *Z* isomers, respectively; and CPA $>$ TBA $\geq$ TDA for *ZE* males and both isomers. Since the differences observed in the EAGs elicited by the isomeric forms of TDA, CPA, and TBA were consistent across strain and mirrored the single sensillum DC potentials elicited by the the isomeric forms of TDA (Hansson et al., 1987), our EAG results indicate the existence of strain differences in detecting modifications in the terminal alkyl group. Although these EAG data are indicative, single sensillum recordings accompanied by cross-adaptation studies are needed for confirmation.

Since sexual behavior in *ZZ* and *ZE* males is elicited preferentially by a pheromone blend with a higher content of (*Z*)-TDA than (*E*)-TDA and the reverse is true for *EE* males (Klun and Maini, 1979), one might expect a similar relationship among the EAG responses if EAG amplitude accurately reflects

behavior (Kaissling, 1971; Roelofs, 1977). A good correlation between our EAG amplitude data and the upwind flight data of Schwartz et al. (1989) was observed for *ZZ* and *ZE* males, but not for *EE* males (Figure 6). This correlation was 0.86 for *ZZ* males ($P > |r| < 0.05$; $N = 6$) and 0.8 for *ZE* males ($P > |r| < 0.04$; $N = 4$); i.e., the upwind flight and EAG responses elicited from *ZZ* and *ZE* males by *Z* isomers were greater ($P < 0.01$) than the responses elicited by the *E* isomers (Figure 6). In contrast, for *EE* males the *Z* isomer

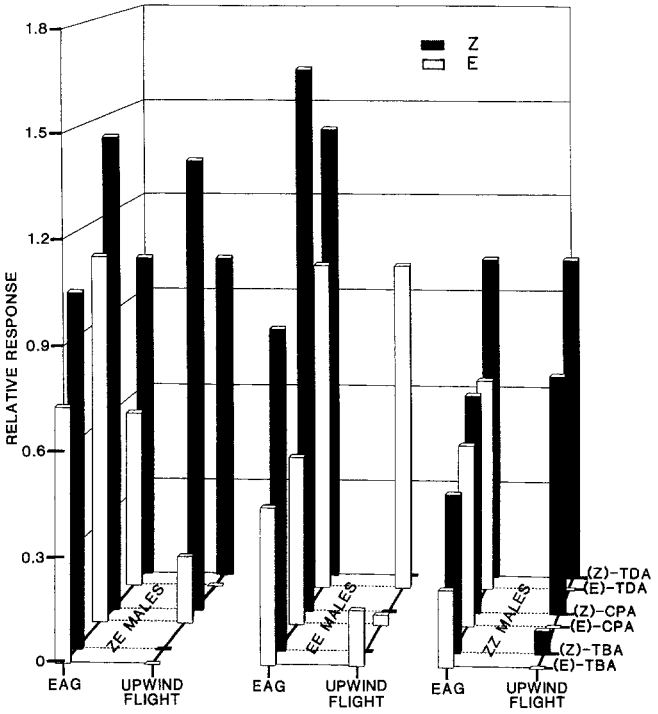

FIG. 6. Relative upwind flight and EAG responses of male *O. nubilalis* to the female sex pheromone and analogs. Within a strain, bars represent values relative to the responses produced by (*E*)-TDA for *EE* males and (*Z*)-TDA for *ZZ* and *ZE* males, separately. The relative within-strain upwind responses to the natural pheromone blend were 1.00, 1.10, and 1.44 for *ZZ* (3:97), *EE* (97:3), and *ZE* (65:35; *E*:*Z*), respectively. The flight tunnel data are from Schwarz et al. (1987) or J.A. Klun (personal communication). Amount of compound used in all behavioral experiments shown was 100 ng. The EAG response compared was elicited by amounts (40 μg for TDA, 80 μg for CPA, and 72 μg for TBA) which produced the maximum amplitude (Figure 2), although similar trends were observed for other amounts on linear portions of the ascending dose–response curves (10–40 μg for TDA, 20–80 μg for CPA, and 18–72 μg for TBA; Figure 2).

elicited a greater EAG amplitude than those elicited by the *E* isomer, which was not correlated with upwind flight (Figure 6). A general trend observed for all strains was the ability of the antennae to detect or elicit EAG responses even when little or no upwind flight was observed (Figure 6).

The inconsistent correlation between EAG amplitude and behavioral response could be the result of one or more of the following: (1) the analogs bind to the same receptors as TDA, but with a lower affinity; (2) the analogs bind to receptors of nonpheromonal olfactory neurons, which provide an EAG response but no behavior; (3) the analogs bind to the same receptors as TDA, but indiscriminantly on the *Z* and *E* neurons (Hansson et al., 1987), thereby providing the wrong action potential code for behavior; or (4) the central nervous system may be different in each strain such that the sensory pattern that evokes behavior in one strain does not do so in another.

The time for repolarization of the EAG is longer for (*Z*)-TDA than for (*E*)-TDA. This was first observed by Nagai et al. (1977); our results confirm their finding and demonstrate that this relatively long repolarization time is unique to the EAGs elicited in *ZZ* male antennae by (*Z*)-TDA. Although the amplitude of the EAG could not be used to distinguish among male strains, the duration of the repolarization time (e.g., width of the peak at half the peak height) could be used to distinguish live *ZZ* males from the other two strains. Since the EAG technique requires less specialized equipment and is easier to perform than the single sensillum techniques, it could be useful in assessing the relative composition of *ZZ* males in populations.

Stimulation of a *ZZ* male antenna with a particular amount of (*Z*)-TDA resulted in a reduction or elimination of the EAGs elicited by amounts lower than that used in initial stimulus, indicating that adaptation of receptors had occurred. Nagai et al. (1977) also observed adaptation of the EAG response from *ZZ* antennae after repetitive stimulation with (*E*)- or (*Z*)-TDA and found the EAG recovered in ca. 20 min. In our study, the EAG response of *EE* antennae recovered in ca. 20 min, which was ca. 10 min faster than the EAG response of *ZZ* antennae. By comparison, *Bombyx mori* (L.) required hours to completely disadapt from a strong stimulus (Kaissling, 1977; Mayer and Mankin, 1985). In *Epiphysas postvittana* (Walker), Rumbo (1988) found most of the adaptation was brought about by a single exposure to a compound [e.g., 10 μg (*E*)-TDA]. We also observed most of the change from the nonadapted state to the adapted state to occur on the first exposure to (*Z*)-TDA (Table 1, Figure 4). Our results indicate that antennal adaptation is strain specific and correlated with the ability of the antenna to repolarize after each stimulus. Repolarization time courses for the EAG response from *B. mori* antennae were observed to be similar to those of the receptor potentials of individual olfactory receptor neurons (Kaissling, 1974). The physiological basis for EAG adaptation is currently hypothesized to be the adaptation of the receptor potential of individual olfactory receptor neu-

rons (Mayer and Mankin, 1985). Thus, the strain-specific differences we observed in the repolarization and disadaptation of the EAG response from *O. nubilalis* antennae could have as its cause: (1) lowered affinity of the receptor for the pheromone molecule, (2) decreased rate of receptor neuron activation, (3) decreased rate of pheromone molecule degradation, and (4) reduction of conductance in the receptor neuron membrane (Kaissling, 1977).

Studies of the effect of adult age on the EAG response showed that peak response occurred during the second day after eclosion. Maximum upwind flight response also occurs on the second day after eclosion (J.A. Klun, personal communication), indicating that the maturation of behavioral responses is linked to that of the sensory cells in *O. nubilalis*. The decline in the EAG response after day 2 implies that the olfactory cells on the antennae are not physiologically stable and may be senescing. Maturation and senescence of the EAG response after emergence was also reported for male and female *Pseudaletia unipuncta* (Haworth) (Seabrook et al., 1979). However, the EAG response of male *Trichoplusia ni* (Hübner) (Payne et al., 1970) and *Argyrotaenia velutinana* (Walker) (Roelofs and Comeau, 1971) showed no change with adult age after emergence. In *Manduca sexta* (L.), maturation of the EAG response and complete development of olfactory sensilla, neurons, and synapses occurs before adult eclosion (Schweitzer et al., 1976; Tolbert et al., 1983). This variation among species in the maturation and senescence of the EAG response suggests comparable differences in the developmental rates of the sensory cells in olfactory systems.

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ANTIFOULING AGENTS FROM MARINE SPONGE *Lissodendoryx isodictyalis* Carter

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Abstract—The sponge *Lissodendoryx isodictyalis* is an odorous, encrusting, blue-gray sponge found on subtidal flats in North Carolina waters. The strong odor of *L. isodictyalis*, coupled with the observation that it is rarely overgrown by fouling organisms, suggested that this sponge may produce metabolites with potent antifouling activity. Ethyl acetate extracts of *L. isodictyalis* inhibit larval settlement of the barnacle *Balanus amphitrite* in laboratory assays at 10 ng/ml. Barnacle settlement bioassays of isolated preparative TLC fractions show that *L. isodictyalis* produces at least two pungently scented, antifouling agents with EC_{50} values of less than 85 μg/ml and less than 250 μg/ml, respectively. The most potent agent inhibits settlement at or below a concentration of 400 ng/ml and kills approximately 25% of settlement-stage barnacle larvae at 400 μg/ml. The other agent causes 100% mortality of larvae at concentrations greater than 400 μg/ml and inhibits settlement at approximately 40 μg/ml. These metabolites of *L. isodictyalis* may inhibit overgrowth of the sponge in nature.

Key Words—*Lissodendoryx isodictyalis*, *Balanus amphtrite*, fouling, antifouling agents, marine sponge.

INTRODUCTION

The marine fouling community is composed of a variety of sessile marine organisms including tunicates, hydroids, bivalves, bryozoans, barnacles, polychaete worms, and sponges that live attached to hard subtidal and intertidal surfaces (Sutherland, 1984). Spatial competition, not predation, is the major factor in

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determining structure of the fouling community in North Carolina waters (Sutherland and Karlson, 1977). Invertebrate larvae and algae may settle on and overgrow other organisms. Such overgrowth provides a potential source of mortality; furthermore, fouling organisms may obstruct feeding, compete with their host for food, and increase sedimentation (Stoecker, 1978; Ware, 1984; Rittschof et al., 1985; Gerhart et al., 1988).

A variety of sessile marine invertebrates, especially octocorals, tunicates, and sponges, are rarely fouled by other organisms. These species frequently contain high concentrations of secondary metabolites (Tursch et al., 1978; Goad, 1978; Faulkner, 1977, 1984; Fenical, 1978, 1982). Some of these metabolites possess potent antifoulant activity, and may play an important role in the inhibition of fouling in nature (Stoecker, 1978; Standing et al., 1982; Targett et al., 1983; Ware, 1984; Rittschof et al., 1985, 1986; Keifer et al., 1986; Bakus et al., 1986; Gerhart et al., 1988).

Lissodendoryx isodictyalis Carter (Order Poesilosclerida) is a blue-gray, encrusting sponge commonly encountered in eel grass (*Zostera marina*) beds in Core Sound, North Carolina. The species is found along the coast of the southeastern United States, the West Indies, and Pacific Mexico, with members of the genus distributed throughout the world (Wiedenmayer, 1977; Laubenfels, 1947). Because of its strong, pungent odor, *L. isodictyalis* is commonly known as the garlic sponge.

Lissodendoryx isodictyalis is rarely overgrown by fouling organisms. This observation, coupled with the strong, garlicky terpenoid odor of the sponge, suggests that *L. isodictyalis* could produce metabolites that prevent fouling. Terpenoids have been shown to prevent fouling in laboratory and field situations (Targett et al., 1983; Rittschof et al., 1985, 1986; Gerhart et al., 1988).

Secondary metabolites from sessile marine invertebrates are rarely tested for their ability to inhibit settlement of sympatric fouling organisms. The acorn barnacle, *Balanus amphitrite* Darwin, can comprise a major component of the fouling community in areas where *L. isodictyalis* is found. The objectives of this study were to determine whether *L. isodictyalis* contains metabolites that inhibit the settlement of *B. amphitrite*, to perform bioassay-directed isolation, and to determine the chromatographic mobility and potency of these biologically active compounds.

METHODS AND MATERIALS

Collection. Lissodendoryx isodictyalis was collected from Core Sound near Straits, North Carolina, at depths less than 1 m below mean low tide. Colonies were collected on September 16, 1987. Sponge tissue was cleaned by removing

and discarding material that the sponge had overgrown. Cleaned sponge tissue was either extracted immediately or frozen for later use.

Extraction. Fresh or frozen tissue of *L. isodictyalis* were soaked overnight in four volumes (w/v) of HPLC-grade ethyl acetate at room temperature. The extract was decanted and the sponge reextracted with four volumes of fresh solvent. The extracts were combined and the cloudy solution filtered through Whatman No. 1 filter paper. The filtrate was concentrated to a viscous oil by rotary evaporation. The extract was partitioned between HPLC-grade methylene chloride and deionized water to remove salts. Solvent was removed from the organic layer using a rotary vacuum evaporator, yielding 0.22 g of dark green, viscous oil. The crude extract was stored in the dark at 4°C in methylene chloride at a concentration of 50 mg/ml. For bioassays, aliquots of this solution were removed, the methylene chloride evaporated, and the residue resuspended in aged seawater filtered to remove particles with size greater than 100 kDa.

Isolation of Antifouling Compounds. Preparative TLC was performed using Whatman PK6F plates (20 × 20 cm). Each plate received 25 mg of extract dissolved in 0.5 ml of methylene chloride. Plates were developed in 1:9 acetonitrile–methylene chloride, and visualized with visible light, long-wave UV light, short-wave UV light, and a vanillin/sulfuric acid spray. TLC plates were divided into six zones, designated 0 through 5, which covered the area from origin to solvent front. Each zone was scraped from the plate, and the compounds were eluted from the silica with HPLC-grade methylene chloride. These solutions were then filtered through Whatman No. 1 filter paper and evaporated to dryness. The resultant residues were tested in bioassays for barnacle-settlement inhibition. Zones that were highly active were further purified by preparative TLC as described above and bioassayed a second time. In initial bioassays (Figure 2 below), fractions were tested at concentrations of 10^{-4} g/ml in seawater. Inhibitory zones of the TLC plate were tested at concentrations ranging from 4×10^{-3} g/ml to 4×10^{-8} g/ml, in steps of 10-fold dilution.

Settlement Inhibition Bioassays. Adult individuals of the acorn barnacle *Balanus amphitrite* Darwin were collected from the Duke University Marine Laboratory seawall in Beaufort, North Carolina. Collected specimens were crushed, and the nauplius-stage larvae they released were cultured to cyprid (settling) stage according to the methods of Rittschof et al. (1984). Settlement assays were performed as previously described by Rittschof et al. (1985). Briefly, 3-day-old cyprid larvae were added to Falcon 50 × 9-mm polystyrene Petri dishes containing either 5 ml of 100-kDa-filtered seawater (approximately 35 ppt salinity) or seawater containing sponge extract. The dishes were incubated at 28°C on a 15:9 light–dark cycle for 22–24 hr. After incubation, the dishes were observed under a dissecting microscope to determine whether the larvae were alive. The larvae were then killed by adding several drops of 10%

formalin. Attached and unattached larvae were counted, and settlement was calculated as the percentage of total larvae that were attached. Differences in the rate of settlement in treatments versus controls were tested for significance using a G test for independence (Sokal and Rohlf, 1981). The number of replicates used in each assay ranged from two to four, depending on the availability of cyprid larvae. The number of replicates was taken into account in statistical analyses of results.

RESULTS

Extraction Yield. Sixty milligrams of crude extract was obtained from 100 g of wet sponge material. The concentration of crude extract within the wet sponge tissue was thus 600 μg/g. The crude extract was dark greenish brown in color and gave off a strong, minty odor. Normal-phase TLC separated the crude extract into 13 distinct bands.

Zone 0 extended from the sample origin to $R_f = 0.10$. Zone 1 extended from $R_f = 0.10$ to 0.20. Zone 2 extended from $R_f = 0.20$ to 0.37. Zone 3 extended from $R_f = 0.37$ to 0.78. Zone 4 extended from $R_f = 0.78$ to 0.90. Zone 5 extended from $R_f = 0.90$ to the solvent front. Compounds from zones 4 and 5 comprised approximately 5% of the total weight of crude extract and were characterized by a greenish color and pungent odor.

Settlement Inhibition by Crude Extracts. Crude extracts significantly inhibited barnacle settlement (Williams adjusted $G = 611$, $P < 0.01$) at concentrations of 10 ng/ml or greater (Figure 1). All larvae were killed at concentrations greater than 400 μg/ml. The EC_{50} for settlement inhibition, estimated graphically, was approximately 100 μg/ml.

Settlement Inhibition by Partially Purified Fractions. Of the six zones generated by preparative TLC, zones 4 and 5 produced the strongest inhibition of barnacle settlement. Zone 2 was moderately active in the settlement inhibition assay, while zones 0 and 1 facilitated settlement (Figure 2). Zone 4 was composed of a green band and an adjacent band that appeared purple under short-wave UV light. Zone 4 possessed an EC_{50} for settlement inhibition that was less than 85 μg/ml (Figure 3). Approximately 25% of the larvae were killed after 24-hr exposure to this compound at concentrations greater than 400 μg/ml. Unkilled larvae appeared less mobile than larvae kept in seawater without additions. The second active component, zone 5, was composed of a yellow band and an adjacent band that also appeared purple under short-wave UV light. This component possessed a settlement inhibition EC_{50} of less than 250 μg/ml (Figure 4). All larvae were killed by zone 5 at concentrations greater than 400 μg/ml.

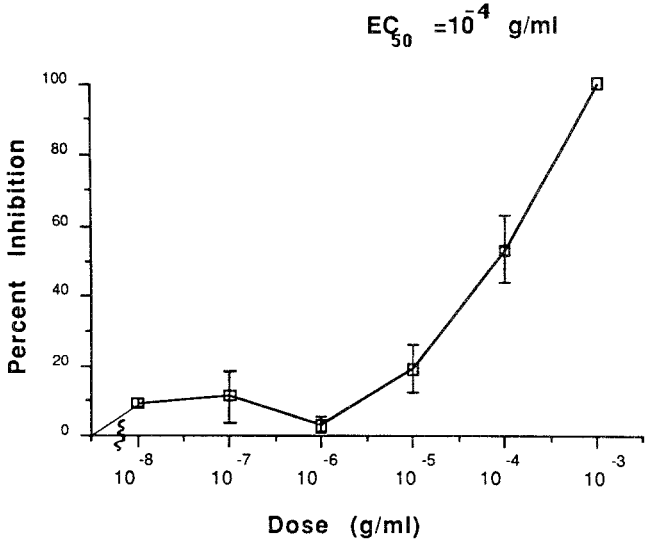

FIG. 1. Inhibition of *Balanus amphitrite* settlement on polystyrene dishes by a crude extract of *Lissodendoryx isodictyalis*. Each rectangle represents the sum of four replicate assays; error bars represent standard errors. Concentrations of 10^{-8} g/ml and higher produced statistically significant inhibition of larval settlement.

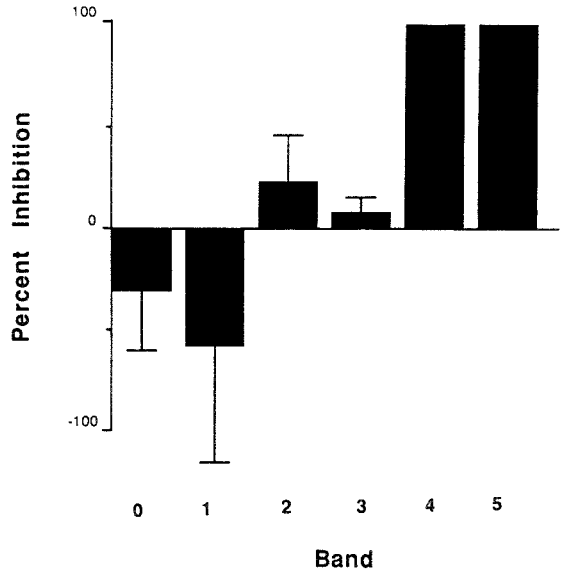

FIG. 2. Inhibition of *Balanus amphitrite* settlement on polystyrene dishes by fractions of *Lissodendoryx isodictyalis* extract at concentrations of 10^{-4} g/ml. Each bar represents the mean of two replicate assays; error bars represent standard errors. Material from zones 0, 1, 4, and 5 produced rates of settlement that differed significantly from control values.

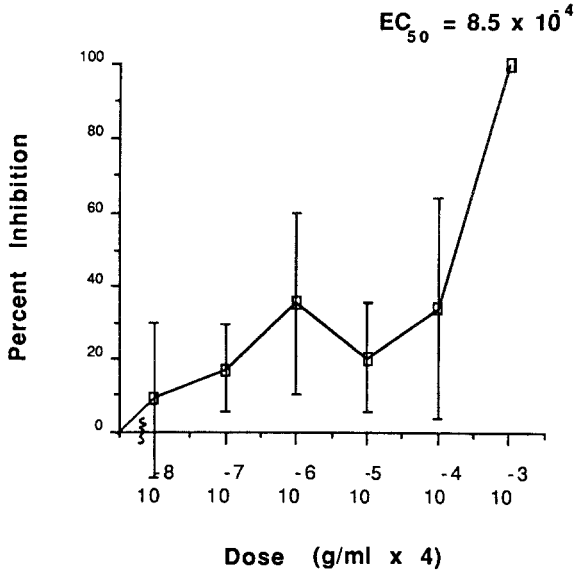

FIG. 3. Inhibition of *Balanus amphitrite* settlement on polystyrene dishes by zone 4 of the *Lissodendoryx isodictyalis* extract. Each rectangle represents the mean of three replicate assays; error bars represent standard errors. Concentrations equal to or greater than 4×10^{-7} g/ml produced statistically significant inhibition of larval settlement.

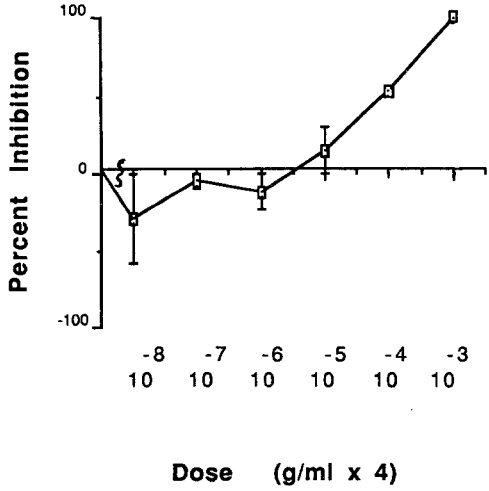

FIG. 4. Inhibition of *Balanus amphitrite* settlement of polystyrene dishes by zone 5 of the *Lissodendoryx isodictyalis* extract. Each rectangle represents the mean of three replicate assays; error bars represent standard errors. Concentrations equal to or greater than 4×10^{-5} g/ml caused statistically significant inhibition of larval settlement.

DISCUSSION

Crude extracts of *L. isodictyalis* significantly inhibit barnacle settlement in the laboratory at concentrations as low as 10 ng/ml. These extracts are similar in potency to the active concentration reported for *Bugula neritina* settlement inhibitors from the sponge *Xestospongia halichondroides* (Ware, 1984). There are at least two antifouling agents in *L. isodictyalis*. Material from zone 4 appears to inhibit settlement by partially anesthetizing the larvae and being somewhat toxic, while fraction 5 appears to prevent settlement by killing larvae. The anesthetizing effect of fraction 4 is similar to that noted for *X. halichondroides* extracts (Ware, 1984). Additional research is needed to determine whether the anesthetizing effect is the result of slowed metabolism, inhibited sensory or motor processes, or other mechanisms.

The antifoulant fractions from *Lissodendoryx isodictyalis* are less potent than purified settlement inhibitors from the octocorals *Leptogorgia virgulata* (Gerhart et al., 1988) and *Renilla reniformis* (Keifer et al., 1986; Rittschof et al., 1986). The R_f values for the antifoulants from *L. isodictyalis* are much higher than those reported for the settlement inhibitors from octocorals (Rittschof et al., 1985; Keifer et al., 1986). In addition, both active fractions are toxic to barnacle larvae, while those from octocorals are not (Rittschof et al., 1985; Keifer et al., 1986). Both characteristics indicate that the compounds from the sponge are different than those previously reported from the octocorals. Further research is in progress to determine the structural identity of these active agents.

The distinctive, minty odor of the inhibitors suggests that the active agents may be terpenoids. Since the pungent aroma of the settlement inhibitors is reminiscent of the odor of the undisturbed sponge in nature, these antifouling agents may be released into the environment by *Lissodendoryx isodictyalis*.

The antifouling activity of *L. isodictyalis* extracts may explain why this sponge is rarely overgrown by fouling organisms in nature. The crude extract comprised 600 μg/g of wet sponge tissue and possessed significantly inhibitory activity at this level. Significant inhibition of barnacle settlement is produced by the most active component of *L. isodictyalis* extracts at levels that are lower than or approximately equal to the tissue concentration (>30 μg/g) of this agent.

The potency of the crude extract may reflect the additive effects of compounds from zone 4. Although the sponge extract possesses components that both inhibit and facilitate settlement of cyprids, the net effect of the sponge crude extract is to inhibit settlement. Similar effects have been noted for extracts of octocorals (Standing et al., 1982). Furthermore, larval settlement occurs only on the surface of the sponge, and surface concentrations of antifouling metabolites may be higher than their concentration as a percentage of the entire col-

ony. Thus, while the EC_{50} values determined in the present study are high compared to those for other antifouling terpenoids, such as pukalide and the renillafoulins (Keifer et al., 1986; Gerhart et al., 1988), the settlement inhibitors from *L. isodictyalis* are still potent enough, relative to natural concentrations within the sponge, to be considered a likely antifouling mechanism. These metabolites may also play multiple roles, acting simultaneously as agents of chemical defense against predators, pathogens, or competitors.

Regardless of their role in nature, the settlement inhibitors from *L. isodictyalis* potentially may provide useful insight into the mechanisms controlling larval settlement and metamorphosis. In addition, nontoxic settlement inhibitors, such as those found in zone 4, are of potential value to marine technology as alternatives to the ecologically damaging toxic chemicals currently incorporated into paints to prevent the fouling of ships.

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ROLE OF PHENOLICS OF CONIFEROUS TREES AS DETERRENTS AGAINST DEBARKING BEHAVIOR OF MEADOW VOLES (*Microtus pennsylvanicus*)

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Abstract—Preferences of meadow voles (*Microtus pennsylvanicus*) toward various coniferous species were tested under winter conditions. Cafeteria tests showed preferences for Norway spruce (*Picea abies*) and Norway pine (*Pinus resinosa*) and rejection of white pine (*Pinus strobus*) and white spruce (*Picea glauca*). When phenolic extracts of these species were prepared and added to a low 8% protein diet, food intake of voles and preferences by rank followed the same patterns. Determination of the concentration of phenolics in branch samples using the Singleton and Rossi procedure showed that Norway pines contained the smallest amount (2.15%/g dry matter) while spruces yielded the most (2.76%/g dry matter). These experiments lead us to expect high losses of Norway pine and Norway spruce in habitats harboring high vole densities.

Key Words—Voles, *Microtus pennsylvanicus*, phenolics, conifers, *Picea*, *Pinus*, deterrents, food choice, preference by rank.

INTRODUCTION

The province of Quebec for several years has sponsored a massive reforestation program aimed at planting 250 million seedlings yearly. Most of this effort is oriented toward conifers because they are easier to handle and they benefit from well-known and standardized growing methods in the nursery. By the time the seedlings are well established in their new habitat, they face various mortality factors, among which meadow voles (*Microtus pennsylvanicus*) are certainly not the least. These small secretive creatures gnaw the base of trunks (Hansson,

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1985) and inflict severe debarking damage when population density is high (Green, 1978). This causes serious disruption in cropping schedules and biomass increment (Sullivan and Vyse, 1987) as well as long-term effects of wounds on tree mortality (Harper and Harestad, 1986).

Small animals like voles and hares are known to be quite selective and usually prefer food items that are low in secondary metabolites (Bryant and Kuropat, 1981; Palo, 1984; Tahvanainen et al., 1985; Bergeron and Jodoin, 1987). This is particularly true of meadow voles because phenolics and tannins are known to reduce food intake (Lindroth and Batzli, 1984), growth, and survivorship when added as supplements to laboratory diets (Lindroth et al., 1986). We have also shown (Bergeron and Jodoin, 1987) negative correlations between food preference indices and levels of total phenolics in herbaceous species. This suggested that their feeding strategy is partly driven by the contents of such secondary metabolites in potential food resources. The bark of trunk and twigs of deciduous trees and conifers contains phenolics and tannins (Bryant, 1981; Sinclair et al., 1982; Tahvanainen et al., 1985), and, since several species of conifers are currently used in reforestation programs, we need to know if the debarking behavior of voles is related to levels of phenolics in young trees and to what extent this can form the basis of their preferences toward particular species under field conditions.

METHODS AND MATERIALS

Wild and laboratory-reared meadow voles were acclimatized to fall and winter conditions in individual polypropylene cages. The mean daily temperature in the first experiment (January 8–14) was $-7.7°C$ and for the replicate (January 21–27), $-0.3°C$. We did not find any significant differences between the two sets of temperature because of the high daily variation that occurred during this period. The enclosure was adjacent to the animal house and roofed to protect the experimental animals from rain, sleet, and snow. They were fed a high-protein (16%) Purina Rabbit Chow, bedded with cotton, and watered ad libitum. Experimental cages measuring $30 \times 60 \times 60$ cm were built with thick plywood (2.0 cm). A layer of 15 cm of potting soil was used to plant seedlings of four conifer species used in this experiment (Norway spruce, *Picea abies*; Norway pine, *Pinus resinosa*; white spruce, *Picea glauca*; white pine, *Pinus strobus*). Roots were protected from gnawing by a floor made of galvanized mesh wire (7-mm mesh size). All conifers used in the cafeteria tests were even-aged ($3\frac{1}{2}$ years) and came from the same public nursery to reduce the biases due to soil type and growing conditions. Prior to each test, one tree per species was planted in each cage and left outside for a week before introducing the animal to its experimental conditions. A ration of 8 g/day of a low 8% protein diet

(ICN diet 904667) and ample bedding and ice cubes were given to voles during the seven-day trial period. The low-protein diet was used to mimic as closely as possible the minimum requirements that voles need to maintain body mass (Lindroth and Batzli, 1984). Cafeteria tests were done on 20 voles but, because of severe winter conditions, a small number of animals died during the tests (five for the first experiment and two during the second). Replicates were performed on all surviving animals, and these were completed by using either wild or laboratory-reared and acclimatized animals to increase sample size.

Daily temperatures and damage level to trees were recorded. Damage indices on individual trees were modified from those of Byers (1974) and Wysolmerski et al. (1980) to meet our experimental procedure. The criteria are summarized as follows: 0 for undamaged trees, 1 for trees with $\frac{1}{3}$ or less of the branches cut, 2 for trees with $\frac{1}{3}$ to $\frac{2}{3}$ of the branches cut, 3 for trees with $\frac{2}{3}$ or more of the branches cut, and 4 when the trunk itself was cut into pieces. These indices were used to rank the species by order of preference. Skewness of data distribution in favor of high-ranking indices forbade the use of parametric tests. Ranks were compared by Kruskal-Wallis tests and ordered by a posteriori multiple comparison tests. Weather data between replicates were compared with Student's t test.

Cafeteria tests were carried a step further using phenolic extracts from conifer seedlings mixed with a low 8% protein diet. Winterized seedlings (without roots and needles) were cut and ground to 1 mm in a Brinkmann mill to prepare phenolic extracts (Singleton and Rossi, 1966). Samples of 66 g of the winterized seedling powder were used for the extractions, which were afterwards mixed in 200 g of the low-protein diet. The mixtures were dried at room temperature for 48 hr, and samples of these were reprocessed through the phenolic extraction method to quantify the real daily dosage given in the rations. The extraction consisted of boiling at 70°C a mixture of ethanol 50% and the powder for 30 min, followed by centrifugation to eliminate the powder. A quantification of total phenolics was done using Folin-Ciocalteu reagent and compared with a standard of gallic acid. Cafeteria tests were conducted under a laboratory standard environment (20°C, 16:8 light–dark regime, 50% relative humidity). They consisted of giving voles a choice between five diets in five different feeding trays: a control diet made of the 8% protein chow and the four experimental ones made of their respective conifer extracts. A ration of 9 g (equivalent to plant extracts from one tree) per experimental tray was given to 10 adult males and 10 adult females to verify daily food intake, which was transformed afterwards into ranks of preferences. Pretest trials on animals fed the control diet for 11 days indicated that the position of feeding trays did not influence food intake.

Student's t test was used to verify body mass differences of experimental animals at the start. Ranks (based on food intake) were analyzed by Kruskal-

Wallis and classified a posteriori by multiple comparison range tests. A Friedman test (K sample test for location-matched data) between data sets of males and females was made to pool the data when necessary. All data sets were processed through the Statistical Analysis System (SAS, 1985).

RESULTS

Since weather conditions can influence food intake in small animals such as voles, we compared the means of daily temperatures between replicates. Both data sets were found to be normally distributed and did not differ significantly (Student's t test = 1.64). It became evident very early in the feeding trials that two coniferous species ranked high as preferred food items, while two others were left almost untouched. Norway pine and Norway spruce were always heavily damaged while white pine and white spruce seemed to be rejected by voles or attacked at a much later date in the experiment. Data sets of the former two species then were compared to those of the last two species using a Kruskal–Wallis test on ranks (Table 1). Both data sets were highly different ($P < 0.05$) with Norway pine and Norway spruce showing the lowest ranks, which means the highest preferences. We then extracted phenolics from branch samples of three of these species to compare the contents between preferred and rejected categories (Table 2). Dosages varied from 2.15%/g dry matter in Norway pine to 2.76%/g dry matter in white spruce. Thus, preferred species had significantly less total phenolics than rejected ones.

We designed a second series of cafeteria tests under more standardized indoor conditions to associate the types of phenolic extracts to preferences (by ranks) among voles. Phenolic additives in diets were also standardized by using an equal quantity of plant powder for each species and incorporating them into

TABLE 1. PREFERENCES OF MEADOW VOLES TOWARD FOUR CONIFEROUS SPECIES AS SHOWN BY KRUSKAL–WALLIS TESTS ON AVERAGE RANK

Experimental features		Rank of preferred species: Norway pine Norway spruce	Rank of rejected species: white pine white spruce
First experiment	Mean rank	14.08[a]	20.96
	N	19	14
Replicate	Mean rank	18.56[a]	31.35
	N	27	20

[a] Found to be significantly different by Kruskal-Wallis tests at $P < 0.05$.

TABLE 2. PHENOLIC CONTENTS OF WHOLE BRANCH AND DIET SAMPLES GIVEN TO VOLES IN CAFETERIA TESTS

Coniferous species	Dosages in whole branch samples from winterized seedlings (mean ± SD)	Dosages in diet samples after laboratory procedures (mean ± SD)
Norway pine	2.15 ± 0.03	0.47 ± 0.03
Norway spruce	2.48 ± 0.04	0.67 ± 0.03
White spruce	2.76 ± 0.08	0.62 ± 0.02
White pine		0.38 ± 0.002

200 g of a low 8% protein diet. Dosages of phenolics from such diets varied from 0.38% per g dry matter in white pine-based diets to 0.67% per g dry matter in Norway spruce based diets (Table 2). This represented only a fraction of the dosages found in branches and shows the impact of laboratory dilution procedures on such compounds.

Mean body mass of experimental males (59.1 g) was not statistically different from that of females (51.0 g: Student's t test = 1.62, $P > 0.05$). Food preferences based on intake data according to time were transformed into a ranking order before being submitted to a Kruskal–Wallis test followed by a posteriori multiple range test. Both males and females rank the experimental diets in a similar manner (Table 3). They preferred, equally well, the control diet and the rations containing Norway pine additives, while they showed a low preference toward white pine and white spruce based diets. A Friedman test between the preferences of males and females was not statistically significant and permitted us to pool the data sets from both sexes and analyze them as one

TABLE 3. CLASSIFICATION OF PREFERENCES BY RANK OF MEADOW VOLES FED DIETS WITH PHENOLIC ADDITIVES[a]

Diet category	Mean rank of males	Mean rank of females	Mean rank from pooled data
Control	5.0	4.5	9.0
Norway pine	18.8	13.5	31.7
Norway spruce	19.1	21.6	40.2
White pine	35.0	30.4	64.8
White spruce	37.1	32.5	69.3

[a] Values connected by vertical bars are not statistically different ($\alpha = 0.05$) (Kruskal–Wallis).

sample (Table 3, right hand side). The control diet that contained no secondary metabolite additives was most preferred, followed by the two diets supplemented with Norway pine and Norway spruce phenolic extracts. Diets with white pine and white spruce extracts had the lowest rank (highest order in the test).

DISCUSSION

Food preferences of wild voles are influenced by the phenolics of herbaceous species (Bergeron and Jodoin, 1987). Moreover, reduction in food intake, growth, and survival (Lindroth and Batzli, 1984; Lindroth et al., 1986) have also been associated with the presence of phenolic additives in diets of laboratory-reared animals. When given the choice between various coniferous species or diets supplemented with plant extracts from conifers, voles showed consistent preferences toward low-phenolic diets. The seedlings of Norway spruce and Norway pine contain less total phenolics than white pine and white spruce. This may explain why Norway spruce and Norway pine registered higher damage levels in cafeteria tests than the other two species. We may hypothesize that the repulsive action is done by only one phenolic compound and that its concentration is proportional to the total phenolic concentration in the conifer. This was confirmed by the second series of cafeteria tests using phenolic extracts from seedlings. Diets supplemented with extracts of the same two species were preferred over those with white pine and white spruce additives. Phenolics are generally considered as deterring substances against herbivores because of their complex association with proteins (Whittaker and Feeny, 1971), and voles seem to take advantage of this by preferring food resources with low phenolic contents.

Our study shows the existence of two conifer categories: one with a low and one with a high predation risk by meadow voles. Norway pines are at high risk but are not extensively used in eastern Canadian reforestation programs. However, Norway spruce is also a high-risk species and is being planted, and has been planted in the past, in the hundreds of millions, mostly in the form of seedlings. These seedlings must withstand four or fives vole cycles in the first 15 years after planting in lands reclaimed from agriculture or in rejuvenated woodlots. Seedlings of Norway spruce are also highly preferred as winter forage by snowshoe hares, *Lepus americanus* (Bergeron and Tardif, 1988), so that high losses are to be expected in years of high population densities of either one or both of these species. Norway pine and Norway spruce probably represent easier growing stocks in public and private nurseries, but they also mean heavier losses for private landowners during times of peak densities of small herbivores.

However, white pine and white spruce seem better able to resist the branch cutting or debarking behavior that voles and hares express in winter. These latter species should be the preferred plantation species over Norway pine and Norway spruce in habitats where high population density of both voles and hares are expected.

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EFFECT OF STEAM DISTILLATE EXTRACT OF RESISTANT WILD RICE *Oryza officinalis* ON BEHAVIOR OF BROWN PLANTHOPPER *Nilaparvata lugens* (Stal) (HOMOPTERA: DELPHACIDAE)

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Abstract—Rice plant volatiles extracted as steam distillates significantly affected the behavior of the brown planthopper *Nilaparvata lugens* (Stal). In a choice test, significantly more females settled and fed on tillers of a susceptible rice variety, Taichung Native 1 (TN 1), sprayed with acetone or untreated TN 1 plants than on TN 1 tillers sprayed with the extracts of resistant wild rice *Oryza officinalis*. *O. officinalis* steam distillate was highly toxic to first-instar nymphs of *N. lugens*. Ingestion and assimilation of food by females was significantly reduced on TN 1 plants sprayed with *O. officinalis* distillate compared to TN 1 plants sprayed with acetone. Application of *O. officinalis* distillate on TN 1 plants adversely affected hatchability of eggs of *N. lugens*.

Key Words—Brown planthopper, *Nilaparvata lugens*, Homoptera Delphacidae, *Oryza officinalis*, plant volatiles, wild rice, plant–insect interaction.

INTRODUCTION

The brown planthopper (BPH) *Nilaparvata lugens* (Stal) causes severe damage to susceptible rice varieties by excessive feeding and transmission of virus diseases. It fails to feed, grow, survive, and reproduce adequately on resistant varieties (Pathak et al., 1969). Reduced feeding on resistant varieties was attrib-

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uted, therefore, to either lack of phagostimulants or to the presence of antifeedants.

Sogawa and Pathak (1970) postulated that the resistance of the rice variety Mudgo was possibly due to existence of smaller quantities of asparagine, which stimulates BPH feeding. Transaconitic acid in the nonhost barnyard grass *Echinochloa crus galli* L. acted as a BPH antifeedant (Kim et al., 1976). Soluble silicic acid and oxalic acid isolated from leaf sheaths of rice plants acted as brown planthopper sucking inhibitors (Yoshihara et al., 1979, 1980). This assumption was abandoned when it was found that silicic acid was a general inhibitor occurring in both resistant and susceptible rice varieties (Saxena, 1986). Kawabe et al. (1980) collected pure phloem sap from rice plants by severing the stylets of planthoppers and leafhoppers feeding on rice plants with a laser beam, and the analysis of the exuded sap indicated that sucrose was the main translocate in the phloem.

Saxena and Okech (1985) discussed the role of rice plant volatiles in the resistance of rice varieties to BPH. According to them, rice plant volatiles extracted as steam distillate were not likely to be found within phloem tissue. The odoriferous and volatile nature of volatiles strongly influenced the internal and external environment of the rice plant and the volatiles were thus ecologically significant in BPH resistance. Khan and Saxena (1985) demonstrated that steam distillate extract of a resistant cultivar, when applied to a susceptible cultivar, disrupted the normal feeding behavior of green leafhopper *Nephotettix virescens*. In the present study we investigated the effect of plant volatiles obtained as steam distillate extract from resistant wild rice *Oryza officinalis* on the behavior of BPH.

METHODS AND MATERIALS

Steam distillate of 50-day-old plants of highly resistant wild rice *O. officinalis* (Heinrichs et al., 1985; Velusamy, 1987) were bioassayed against greenhouse-reared *N. lugens*.

Steam Distillation and Extraction of Rice Plant Volatiles. Leaf sheaths of 50-day-old plants of the resistant wild rice *Oryza officinalis* (IRRI accession number 100179) grown in an insect-proof screenhouse were harvested and ground with an electric grinder. Steam distillation and extraction were done according to the method of Saxena and Okech (1985). A 200-g ground sample was steam distilled for 3 hr, during which time approximately 900 ml of distillate were collected. The distillate was extracted with diethyl ether (300 ml distillate; 100 ml diethyl ether) by thoroughly shaking a mixture of the two together in a separating funnel for 5 min. Diethyl ether extracted the essential oils and

other volatiles, and the ether mixture settled above the water layer in the funnel. If emulsion was formed while mixing, a few crystals of sodium sulfate were added to break the emulsion. The water layer was discarded while the ether extract was pooled in a 500-ml beaker, to which 50 g of anhydrous sodium sulfate was added. The resultant mixture was kept inside a fume hood to evaporate excess ether until the remaining volume was approximately 25 ml. The beaker then was covered with aluminum foil and held overnight to allow sodium sulfate to absorb traces of water from the extract. The extract was evaporated further to 10 ml and decanted into a weighed glass vial, which was then covered with perforated aluminum foil and placed inside a desiccator. Ether was evaporated under vacuum, leaving behind a yellow oily residue. The vial was reweighed, sealed with nitrogen, and kept at $-10°C$. The yellow oily residue was diluted in acetone–water (4 : 1) and tested at levels of 1000, 2000, 3000, and 4000 ppm.

Influence of Wild Rice Plant Volatiles on BPH Settling Responses. The settling responses of BPH were tested by the method described by Saxena and Okech (1985). Thirty minutes before exposure to the BPH, single tillers of 30-day-old TN 1 plants grown in 6-cm-diameter pots were sprayed with 1 ml of acetone–water solutions of extracts of resistant wild rice at a rate of 1000, 2000, 3000, or 4000 ppm using a quick spray atomizer. Control plants were untreated or treated with acetone. The pots with treated and control plants were arranged 7 cm apart in a circle in a plastic container (30 cm diameter, 16 cm deep) partly filled with water. The tillers of test plants were inserted through 1.5-cm-diameter holes bored equidistantly near the periphery of the lid covering the container. Fifty newly emerged, CO_2-anesthetized, brachypterous females were released at the center of the lid and covered with a snug-fitting cylindrical Mylar cage (55 cm height, 30.2 cm diameter). The females moved to the plants upon revival. Individuals that settled on each plant were counted at 4, 8, 24, and 48 hr after release. The experiment was replicated five times in a randomized complete block design.

Toxicity of Steam Distillate Extracts to First-Instar Nymphs. Toxicity of steam distillate extracts to first instars was tested following the method of Khan and Saxena (1985). Ten-day-old TN 1 seedlings were dipped separately in 1000, 2000, 3000, or 4000 ppm solutions of extracts of resistant wild rice and placed individually in test tubes (15×1.5 cm). Control seedlings were untreated or treated with acetone. After the acetone had evaporated, 10 first instars were released on each of the treated or control seedlings. The test tubes were capped and arranged in a randomized complete block design in an incubator. Nymphal mortality was recorded after 24 hr. Each treatment, including the control, was replicated five times.

Ingestion and Assimilation of Food on Extract-Treated Plants. Leaf sheath

portions of intact tillers of potted 40-day-old TN 1 rice plants were painted using a camel-hair brush with 0.25 ml of acetone–water solutions of extracts of resistant wild rice at a rate of 1000, 2000, 3000, or 4000 ppm per tiller. Control plants were untreated or treated with acetone. One hour after treatment, newly emerged females (starved for 2 hr but water satiated) were weighed individually on a microbalance and enclosed singly in air-tight parafilm sachets (5 × 5 cm) through which the leaf sheath of treated or control plants passed. The weight of each female and its excreta were recorded separately after 24 hr. A control insect was maintained to assess the loss in insect body tissue as a result of catabolism, and the insect was given access to a moist cotton swab to prevent desiccation. The amount of food ingested and assimilated was calculated as follows (Khan and Saxena, 1986):

$$\text{Food assimilated} = W_1\left(\frac{(C_1 - C_2)}{C_1}\right) + (W_2 - W_1)$$

where W_1 is the initial weight of insect; W_2 is the final weight of insect; C_1 is the initial weight of control insect; C_2 is the final weight of control insect; and food ingested is the food assimilated + weight of excreta. The experiment was replicated 10 times.

Hatchability of Eggs on Treated Plants. Thirty-day-old single tillers of potted TN 1 plants were infested with five gravid brachypterous females and allowed to oviposit for 24 hr after, which the plants were removed and painted with 0.25 ml acetone solutions at a rate of 1000, 2000, 3000, or 4000 ppm extract per tiller. Control plants were untreated or treated with acetone. The number of nymphs that emerged was recorded, and at the end of emergence of nymphs, unhatched eggs were counted by dissecting leaf sheaths under a 20× binocular microscope.

RESULTS

Influence of Steam Distillate Extract on Settling Responses. In the choice test, BPH adults settled uniformly on TN 1 plants sprayed with extract of resistant *O. officinalis* or with acetone 1 and 4 hr after release. However, 8 hr after release, there were significantly fewer females on TN 1 plants sprayed with *O. officinalis* extract than on acetone treated plants (Table 1). Fewer females were also recorded on TN 1 plants sprayed with the extracts of resistant wild rice *O. officinalis* than on control plants even 24 and 48 hr after release.

Toxicity of Steam Distillate Extracts to First-Instar Nymphs. The extracts of *O. officinalis* were highly toxic to first-instar nymphs (Table 2). There were significant differences in toxicity among extracts of different concentrations, and extracts at 3000 and 4000 ppm caused significantly higher mortality of

TABLE 1. SETTLING RESPONSE OF *N. lugens* FEMALES ON TN 1 PLANTS SPRAYED WITH STEAM DISTILLATE EXTRACTS OF RESISTANT WILD RICE *O. officinalis* AT 4, 8, 24, AND 48 hr AFTER RELEASE IN FREE-CHOICE TEST

Cultivar	Treatment	Individuals settled (%) on plants at given hr after release[a]			
		4	8	24	48
TN 1	None	10.4a	22.4a	28.6a	27.2a
TN 1	Acetone	11.2a	21.6a	29.0a	29.4a
TN 1	1000 ppm *O. officinalis*	12.6a	8.2b	6.4b	5.0b
TN 1	2000 ppm *O. officinalis*	12.0a	8.0b	5.6bc	4.2bc
TN 1	3000 ppm *O. officinalis*	13.4a	6.2bc	4.4bc	3.2cd
TN 1	4000 ppm *O. officinalis*	10.8a	5.4c	3.0c	2.6d

[a] Mean of five replications. Means within a column followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test (DMRT).

nymphs on extract-treated TN 1 plants than lower concentrations. Even at 1000 and 2000 ppm, more nymphs died on TN 1 susceptible plants treated with extracts of resistant wild rice than on acetone-treated and control plants. The nymphs rejected the treated plants and settled on Mylar cages.

Ingestion and Assimilation of Food on Treated Plants. The quantity of food ingested and assimilated by BPH was significantly less on extract-treated TN 1 plants than on acetone treated or control TN 1 plants (Table 3). Distinct differences in quantity of food ingested were recorded among different concentrations of extracts, and ingestion was significantly lowest on TN 1 plants

TABLE 2. TOXICITY OF STEAM DISTILLATE EXTRACTS OF RESISTANT WILD RICE *O. officinalis* TO FIRST-INSTAR NYMPHS OF *N. lugens*

Cultivar	Treatment	Nymphal mortality (%)[a]
TN 1	None	0d
TN 1	Acetone	2.0d
TN 1	1000 ppm *O. officinalis* extract	70.0c
TN 1	2000 ppm *O. officinalis* extract	88.0b
TN 1	3000 ppm *O. officinalis* extract	94.0ab
TN 1	4000 ppm *O. officinalis* extract	98.0a

[a] Mean of five replications. Means followed by the same letter are not significantly different at the 5% level by DMRT.

TABLE 3. EFFECT OF STEAM DISTILLATE EXTRACTS OF RESISTANT WILD RICE *O. officinalis* ON *N. lugens* FEEDING AND ASSIMILATION

Cultivar	Treatment	Food (mg) ♀/14 hr[a]	
		Ingested	Assimilated
TN 1	None	11.08a	0.79a
TN 1	Acetone	10.98a	0.78a
TN 1	1000 ppm *O. officinalis* extract	4.60b	0.29b
TN 1	2000 ppm *O. officinalis* extract	2.90c	0.27b
TN 1	3000 ppm *O. officinalis* extract	1.76d	0.23bc
TN 1	4000 ppm *O. officinalis* extract	1.14e	0.20c
O. officinalis	None	0.62f	0.05d

[a] Mean of 10 replications. Means within a column are not significantly different at the 5% level by DMRT.

sprayed with extract at 4000 ppm level. Likewise, the quantity of food assimilated was also significantly lower on TN 1 plants treated with extract at 4000 ppm compared to 1000 and 2000 ppm.

Egg Hatchability on Treated Plants. Application of extracts of *O. officinalis* to TN 1 plants significantly reduced egg hatchability, but application of acetone to TN 1 plants had no effect (Table 4). The percentage of egg hatchability on extract-treated TN 1 plants differed significantly and decreased progressively with application of increased concentrations of *O. officinalis* extract.

TABLE 4. PERCENTAGE EGG HATCH ON *N. lugens* ON TN 1 PLANTS SPRAYED WITH EXTRACT OF RESISTANT WILD RICE, *O. officinalis*

Cultivar	Treatment	Percentage egg hatch[a]
TN 1	None	96.03a
TN 1	Acetone	95.55a
TN 1	1000 ppm *O. officinalis*	53.99b
TN 1	2000 ppm *O. officinalis*	43.48c
TN 1	3000 ppm *O. officinalis*	36.07d
TN 1	4000 ppm *O. officinalis*	32.27e
O. officinalis	None	28.78f

[a] Mean of five replications. Means followed by a common letter are not significantly different at the 5% level by DMRT.

DISCUSSION

Plant chemicals are very important in insect–plant relationships and even subtle differences in the physical and chemical attributes of plants can affect their suitability as hosts. Recently, several authors have demonstrated the role of plant chemical factors in determining susceptibility of rice cultivars to insect pests (Pathak and Saxena, 1980; Khan and Saxena, 1985; Saxena and Okech, 1985; Saxena, 1986). The present study elucidated the role of rice plant volatiles as allelochemics (Whittaker and Feeny, 1971) involved in BPH–rice plant interactions. The low settling response of BPH females on TN 1 plants treated with steam distillate extracts of highly resistant *O. officinalis* plants showed that the treatment conferred resistance at least temporarily. Similarly, nymphs caged on treated TN 1 plants were unable to settle on them and suffered high mortality. The restlessness of BPH nymphs and adults on treated susceptible TN 1 plants can be attributed to the volatile compounds present in the distillate, which are deleterious to the insect (Saxena and Okech, 1985).

Food intake and assimilation also decreased significantly in susceptible TN 1 plants treated with extract of *O. officinalis*. TN 1 plants treated with *O. officinalis* extract at 4000 ppm conferred a high level of resistance against insect feeding. Khan and Saxena (1986) also demonstrated that ingestion and assimilation of food by *Sogatella furcifera* females were reduced more on TN 1 plants treated with extracts of resistant varieties than on plants treated with acetone or TN 1 extracts. According to them, the volatile repellents present around resistant plants would discourage prolonged staying and sustained feeding of insects on them and therefore could have a multitude of subtle, yet distinct effects on pest behavior and could limit pest establishment.

Reduced egg hatchability on TN 1 plants treated with extract *O. officinalis* compared with acetone-treated or control TN 1 plants suggests that the extract of *O. officinalis* has an allelochemical (or allelochemicals) that affects egg hatching. Previous studies have shown that topical application of steam-distillate extract of resistant TKM 6 on eggs of striped stem borer, *Chilo suppressalis*, adversely affected embryonic development and reduced egg hatchability. Saxena and Puma (1979) also demonstrated that hatchability of BPH eggs was greatly reduced when incubated for three days in 0.5% transaconitic acid, an allelochemic factor present in *Echinochloa crusgalli* L.

The allelochemicals in the resistant and susceptible rice cultivars have not been identified but a large group of low-molecular-weight compounds, such as essential oils, terpenoids, alcohols, aldehydes, fatty acids, esters, and waxes have been obtained (Gunther, 1952; Bianchi et al., 1979). A mixture of 14 esters, seven carbonyl compounds, five alcohols, and isocyanurate in the volatile fraction of BPH-susceptible rice cultivar has been identified (Obata et al.,

1985). Saxena and Okech (1985) detected 34 different peaks in the steam distillate extracts of different rice cultivars. Most of these compounds are insoluble in water and are not likely to be translocated in the vascular bundles.

Sogawa (1982) suggested the occurrence of sucking inhibitory chemicals in the phloem sap of resistant varieties. However, our studies have demonstrated that application of steam-distillate extract of resistant varieties to susceptible plants can confer resistance against BPH without altering the chemistry of phloem sap in susceptible plants. It has also been demonstrated for another rice green leafhopper, *N. virescens* (Khan and Saxena, 1985), that resistance of susceptibility to feeding was governed by the sum of allomones or kairomones present in rice plant volatiles. Thus, our findings support Fraenkel's (1959, 1969) view that allelochemics play an important role in insect–plant interactions.

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IDENTIFICATION AND SYNTHESIS OF INSECT PHEROMONE
XXVIII. Sex Pheromone of Poplar Pole Clearwing Moth, *Sphecia siningensis* Hsu[1]

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Abstract—(*Z*,*Z*)-3,13-Octadecadien-1-ol **1a** is identified as the sex pheromone from the poplar pole clearwing moth, *Sphecia siningensis* Hsu, by GC-MS analysis, synthesis, EAG, and field bioassay. Species isolation among Sesiidae by pheromone is also discussed.

Key Words—Poplar pole clearwing moth, *Sphecia siningensis* Hsu, sex pheromone, Lepidoptera, Sesiidae, (*Z*,*Z*)-3,13-octadecadien-1-ol, geometric isomers.

INTRODUCTION

The poplar pole clearwing moth, *Sphecia siningensis* Hsu was once named poplar large hornet moth *Aegeria apiformis* Clerk (Hsu, 1981). This pest is distributed over Shanxi, Gansu, Qinghai, Ningxia, Shanxi, Inner Mongolia, and other provinces in China (Zhong, 1983; Lin, 1984), which are high elevation, dry, cold areas, covering an area of 26,700 ha. It damages trees over 8

[1] Studies on the identification and synthesis of insect pheromone XXVII. Stereoselective synthesis of (5*R*, 6*S*)-(−)- and (5*S*, 6*R*)-(+)-mosquito oviposition attractant pheromone and stereochemistry of asymmetric addition of a chiral sulfoxide to a chiral aldehyde. Zhou, W.S., Cheng, J.F. and Lin, G.Q., *Acta Chemica Sinica* 46:274, 1985.

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years old by boring into the base or bole and sometimes even into branches. Under severe attack, the xylem and pith are extensively damaged and are easily snapped by a strong wind.

The poplar pole clearwing moth has one generation every two years (Zhong, 1983; Hsu, 1984). Larvae develop in the tunnel of the tree for 23 months; adults, eggs, and larvae live outside the tree pole for a few days. Therefore, it is very difficult to control. Since it mates only once, mass trapping using the sex pheromone could be used for control. As we previously reported (Zhang et al., 1986), in the field tests, one of the synthetic isomers **1a**-(*Z*, *Z*), although inactive toward *Paranthrene tabaniformis* Rott, the poplar twig clearwing moth, was highly attractive to *Sphecia siningensis* Hsu. Both of them belong to Sesiidae. We report here the structure determination, synthesis, and pheromone response by the poplar pole clearwing moth *S. siningensis* Hsu, as well as species isolation among Sesiidae.

METHODS AND MATERIALS

Pheromone Collection. Moths were collected from the countryside of Yuci City, Shanxi Province, and 2-day-old unmated male moths were used for EAG tests. The ovipositor areas of 1- to 2-day-old unmated females (80 FE) were dipped and extracted with CH_2Cl_2. The extract was filtered over glass wool, and the filtrate was concentrated under dry N_2 flow to 20 μl for analysis.

Instruments. For the natural product, MS spectra were obtained on a Shimadzu QP-1000 GC-MS instrument equipped with 3% OV-1 column (2 m $\times$ 2 mm). The temperature was programmed from 150°C (1 min) to 250°C at 5°C/min. For the synthesized compounds, [^{1}H]NMR spectra were recorded in $CDCl_3$ solution with TMS as an internal standard on Varian XL-200 and EM 360L spectrometers. Capillary gas chromatographic analyses were performed on HP 5880A-type instrument equipped with a DEGS column (40 m $\times$ 0.28 mm) at a column temperature of 180°C with a split ratio of 40:1. MS spectra were obtained with Finnigan 4021 model spectrometer with an EI source.

Bioassay. EAG equipment was constructed according to Roelofs and Comeau (1971). The chemicals were diluted with CH_2Cl_2 to 1 mg/ml and transferred (10 μg) onto a piece of filter paper in a 1-cm^2 area with a microsyringe. Every sample was tested no more than three times on each antenna in random order; all tests were performed in a well-ventilated laboratory.

Field tests were conducted in the forest belt (trees 10–12 years old) in Taigu County of Shanxi Province and Dingxi and Ninxia regions of Gansu Province during the adult flight seasons from 1983 to 1987. Delta-type adhesive traps were used and hung on trees about 1.5 m above the ground. Traps were charged with various amount of synthetic lure using red rubber specta as dispensers.

RESULTS AND DISCUSSION

Synthesis.

1a, *Z,Z* R = H

1b, *E,Z* R = H

1c, *Z,E* R = H

1d, *E,E* R = H

$$n\text{-}C_4H_9CH\overset{13}{=}CH(CH)_8CH\overset{3}{=}CH(CH_2)_2OR$$

2a, *Z,Z* R = Ac

2b, *E,Z* R = Ac

2c, *Z,E* R = Ac

2d, *E,E* R = Ac

(*Z,Z*)-3,13-Octadecadien-1-ol **(1a)**, its geometric isomers **(1b–d)**, and the corresponding acetate **(2a)** were prepared as previously reported by Zhang et al. (1986) according to the following scheme. The geometric purities of **1a–d** and **2a** were all more than 95% and these were employed in EAG and field tests.

The procedure chosen to prepare **1a** is outlined in Scheme 1. The preparation of compounds **3–10** was done according to our previous report (Zhang et al., 1986). Treatment of **10** (84 g) with TsOH—CH_3OH followed by P-2 Ni

$$HC{\equiv}CCH_2OH \xrightarrow[80\%]{a} C_7H_{15}C{\equiv}CCH_2OH \xrightarrow[83\%]{b} HC{\equiv}C(CH_2)_8OH \xrightarrow[83.6\%]{c,d}$$

3 **4** **5**

$$C_4H_9C{\equiv}C(CH_2)_8OTHP \xrightarrow{e} C_4H_9\overset{H}{C}{=}\overset{H}{C}(CH_2)_8X \xrightarrow[94\%]{h}$$

6

f, g: **7** X = OTHP; **8** X = OH; **9** X = Br

$$C_4G_9C{\equiv}C(CH_2)_8\overset{H}{C}{=}\overset{H}{C}(CH_2)_2OTHP \xrightarrow[90\%]{f,e} C_4H_9\overset{H}{C}{=}\overset{H}{C}(CH_2)_8\overset{H}{C}{=}\overset{H}{C}(CH_2)_2OH$$

10 **1a**

SCHEME 1. (a) Li-Liq.NH_3, *n*-$C_7H_{15}Br$; (b) NaH-1,2-PDA or 1,2-EDA; (c) DHP, HCl; (d) *n*-BuLi/*n*-BuBr; (e) P-2 Ni/H_2; (f) TsOH—CH_3OH; (g) Py-TsCl LiBr-acetone; (h) LiC≡CCH_2OTHP-HMPT.

(Brown and Ahuja, 1973) gave **1a** (75.6 g) in 90% yield. The overall yield of **1a** from **3** was 43%. Calcd. for $C_{18}H_{34}O$: C, 81.13, H, 12.86. Found: C, 81.00, H, 13.18. IR, ν_{max}: 3300 (OH), 3010 and 730 (*cis*-CH=CH) cm^{-1}. NMR, δ_H: 0.83 (3H, t = 7, CH_3), 1.26 [16H, br, (CH_2)n], 1.47–2.3 (8H, m, CH_2C=), 3.60 (2H, t, J = 7, $C\underline{H}_2OH$), 5.24–5.6 (4H, m, CH=CH) ppm. MS, *m/z*: 266 (M^+, 1.6%), 248 ($M-H_2O$, 0.5%), 55 (base). GC (DEGS) analysis showed that **1a** contained *Z,Z*, 95.2%; *Z,E*, 2.7%; *E,Z*, 1.0%; and *E,E*, 1.1%, with R_t of 27.68, 26.36, 25.25, and 24.12 min, respectively.

The other three isomers **(1b–d)** were synthesized in a similar manner to **1a** (Zhang et al., 1986) and were fully characterized by IR, NMR, and MS spectrometry. **1b:** *E,Z*, 95.1%; *E,E*, 2.9%; *Z,Z*, 1.1%; *Z,E*, 0.9%; **1c:** *Z,E*, 95.2%; *E,E*, 3.5%; *Z,Z*, 1.3%; **1d:** *E,E*, 95%; *Z,Z*, 3%; *Z,E*, 1%; *E,Z*, 1%.

Structural Determination. The pheromone extract (2 μl) was injected into a GC-MS for mass fragmentography using *m/z* 266 (M^+) and 248 (M-18). There was a peak on GC (Figure 1) with R_t 16.1 min, which was the same as that of synthetic **1a** under the same conditions and could be detected at both *m/z* 266 (M^+) and 248 (M-18). The MS spectra obtained from this peak was shown to be identical to that of **1a** (Figure 2).

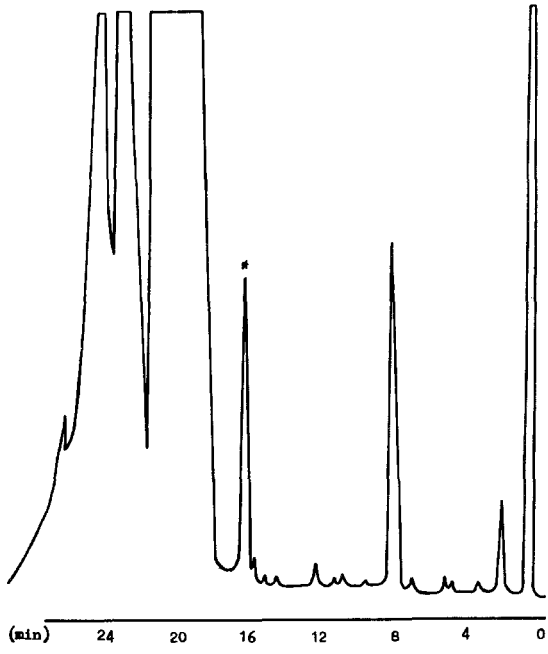

FIG. 1. Gas chromatogram of the pheromone gland washings from *Sphecia Siningensis* Hsu. GC condition: column 3% OV-1, column temperature programmed from 150°C to 250°C at a rate of 5°C/min.

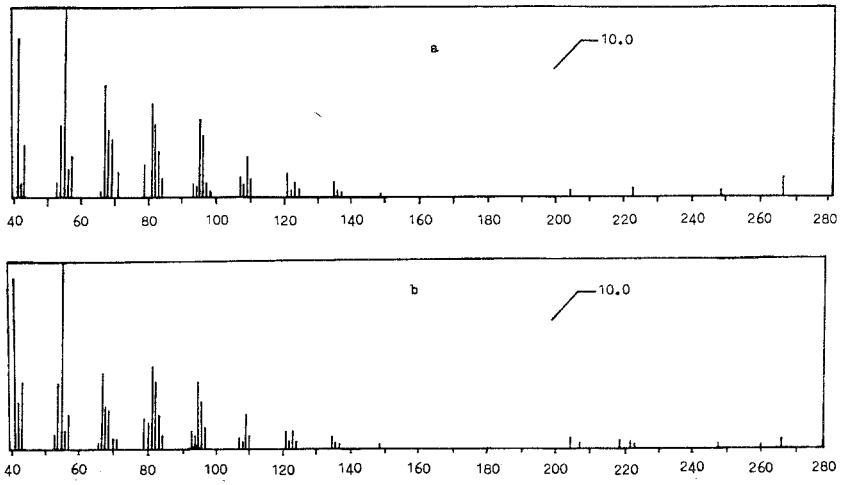

FIG. 2. MS spectra of natural pheromone of *Sphecia siningensis* Hsu (b) and the synthetic compound **1** (a).

Further evidence was obtained by bioassays. Among eight compounds tested, (*E*,*E*)-OH[5] **(1d)** elicited the highest response to male moth antenna, (*Z*,*Z*)-OH **(1a)** is the second highest. The other two isomers **(1b, 1c)** and all the corresponding acetates **(2a–d)** have no effect in the EAG bioassay (Table 1). The average response for **1a** was 1.25 mV (75.76%, in comparison with that of **1d** as 100%), and for **1d** it was 1.65 mV. The average responses for **1b** and **1c** were 0.15 mV and 0.20 mV, which might be due to contamination. The related acetates **2a–d**-OAc and the control test has no detectable signal on EAG. Besides, in each EAG test **1d** always got the highest response, **1a** the second highest.

However, in the field tests only **1a** was attractive to male moth. Although (*E*,*E*)-OH **(1d)** had the strongest EAG reaction by the poplar pole clearwing moth, it acted as an inhibitor in field trials; therefore it seems that there are two kinds of chemoreceptors in antennae. Compound **1d** stimulates the inhibitional chemoreceptor in antennae and produces an EAG reaction. Compound **1a** stimulates the sex pheromone receptor and is responsible for sex behavior.

The same effect was observed in the case of the EAG response test of the male poplar twig clearwing moth. *P. tabaniformis* Rott where both **1a** and **1b** gave strong EAG responses; the other two isomers, **1c** and **1d**, gave rather weak responses. However, in the field bioassays, only **1b** proved to be the natural pheromone. Thus **1a** was established as the sex pheromone of the poplar pole clearwing moth.

[5](*E*,*E*)-OH stands for (*E*,*E*)-3,13-octadecadienol; similar abbreviations were used for the other compounds.

TABLE 1. EAG RESPONSE OF POPLAR POLE CLEARWING MOTH TO DIFFERENT CHEMICALS

Chemical samples	Amplitude of reaction of EAG (mv)					EAG (%)
	I	II	III	IV	$\bar{X}$	
1a, *Z,Z*-OH	2.0	1.8	0.8	0.4	1.25	75.76
1b, *E,Z*-OH	0.4	0.2	0	0	0.15	9.20
1c, *Z,E*-OH	0.4	0.2	0.2	0	0.20	12.27
1d, *E,E*-OH	3.0	2.3	1.0	0.2	1.65	100
2a–d-OAc	0	0	0	0	0	0
CK	0	0	0	0	0	0

Field Evaluation. In an observation spot, the dose of each trap was 200 μg and the test was repeated twice. During the peak period of adult emergence, a number of male moths flew toward the trap baited with (*Z,Z*)-OH at 8 PM. Males circled the traps for 1–2 min, then entered it. Trap capture averaged 50 males per day. The other geometric isomers were ineffective in attracting males.

To compare the trapping efficiency of different dose of Z,Z-OH **(1a)** to male moths, four separate experiments were done in three different locations in 1986–1987. Table 2 shows that among the doses of 200, 400, and 800 μg, those with dose of 800 μg caught the most males. For example, in Taigu, 16 traps with a dosage of 800 μg caught 623 male moths during August 15 to September 30 with an average 39 moths per trap, while those with dosages of 200 μg and 400 μg caught an average of 20.5 and 23.4 moths per trap.

In Table 3, six traps of different doses were used, and the number of captured males was recorded each day. The trap with a dosage of 800 μg caught

TABLE 2. COMPARISON OF PHEROMONE RESPONSE BY POPLAR POLE CLEARWING MOTH TO DIFFERENT TRAP DOSES AT THREE LOCATIONS

Place	Date[a]	200 μg			400 μg			800 μg		
		Traps	Moths	$\bar{X}$	Traps	Moths	$\bar{X}$	Traps	Moths	$\bar{X}$
Taigu	Aug. 15–Sept. 30*	16	328	20.5	16	375	23.4	16	623	39.0
	Aug. 15–Sept. 30**	36	271	7.5	36	339	9.4	36	382	10.6
Dingxi	Aug. 3–28*	29	63	2.2	81	285	3.5	54	233	4.3
Linxia	Aug. 25–Sept. 4**	4	11	2.8	4	62	15.5	4	74	18.5

[a]The experiments were done in *1986 and in **1987.

TABLE 3. RESPONSE OF POPLAR POLE CLEARWING MOTH TO DOSES OF (*Z*,*Z*)-3,13-OCTADECADIEN-1-OL IN TRAPS AT LONGXI COUNTY, GANSU PROVINCE, 1986

Date	Moth caught at 200 μg	400 μg	800 μg	1200 μg	1600 μg	1800 μg	2000 μg	Σ *X*
Aug. 11–13	4		71		8			83
Aug. 14–16		14	30	27	22			93
Aug. 17–19								
Aug. 20–22			96	12				108
Aug. 23–25			9					9
Aug. 26–28			18					18
Total	4	14	224	39	30	0	0	311
Percentage of total	1.29	4.5	72.0	12.54	9.6	0	0	100

the largest number of male moths, which was 72% of the total number of captured male moths.

The trap baited with 800 μg synthetic pheromone was five to seven times more attractive than that of the trap with dose of 1200 μg of the total number of the captured male moths. When the dose used was over 1600 μg, no moths were caught; the male moths only flew near the trap instead of entering it. For convenience, traps at dosages of 600–800 μg were used in the field to get optimum catches.

Species Isolation among Sesiidae. In order to understand the species isolation by pheromone, two more species of clearwing moths in China were examined by screening test in the field. Male *Paradoxecs prelli lien*, a mulberry tree borer, gave strong response only to **2b,** an acetate of (*E*,*Z*)-3,13-Octadecadien-1-ol (Tan, unpublished), and the related vine tree borer *Paranthrene regalis B* was captured by **1b** (Guo et al., unpublished). Therefore, two kinds of clearwing moth use the same *E*,*Z*-OH **(1b)** as the major pheromone component. There must be either a minor component in the pheromone of one of the species or other reasons for the species isolation. This is now under investigation.

CONCLUSION

The structural determination in combination with EAG and field bioassays gave strong evidence that (*Z*,*Z*)-3,13-Octadecadien-1-ol **(1a)** is a sex pheromone of the poplar pole clearwing moth *Sphecia siningensis* Hsu. Other geo-

metric isomers **(1c–d)** and the corresponding acetates **(2a–d)** were inactive. Compound **1a** demonstrated a high level of attractancy to the poplar pole clearwing moth, which could be useful for mass trapping the male moths or for monitoring adult emergence in pest control programs.

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CHEMICAL AND BEHAVIORAL STUDIES ON DUFOUR GLAND CONTENTS OF *Manica rubida* (HYMENOPTERA: FORMICIDAE)

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Abstract—The Dufour gland of *Manica rubida* contains a simple mixture of (*Z*, *E*)-α-farnesene and (*Z*, *E*)-α-homofarnesene with tiny amounts of other farnesene, homofarnesene, and bishomofarnesene isomers. The gland also contains a mixture of very volatile compounds, chiefly acetone, with smaller amounts of acetaldehyde, ethanol, propanol, isobutyraldehyde, butenone, and butanone. The workers of *M. rubida* are not attracted to these highly volatile compounds as are workers of *Myrmica* species, but *M. rubida* workers show a strong increase of linear speed when stimulated by a freshly isolated gland. This response of increased speed of both *M. rubida* and *M. rubra* to their own or each other's Dufour gland secretion is consistent with the presence of the farnesenes in them both.

Key Words—Ant, *Manica rubida*, Hymenoptera, Formicidae, Dufour gland, farnesene, homofarnesene, acetone.

INTRODUCTION

Manica rubida (Latreille) is the only Eurasian member of the genus *Manica* Jurine. It is generally described as a primitive myrmicine ant on the basis of its

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colony foundation system and some of its morphological characters. Moreover, it does not recruit nestmates to collecting food. Goetsch (1934) has already shown that *Manica rubida* workers lay odor trails. We have observed that foragers lay down trails on their way back to the nest, then again to the food, and follow these trails singly (M.C. Cammaerts and Cammaerts, 1985; R. Cammaerts and Cammaerts, 1985). The trail pheromone is a minor component of the venom contained in the poison gland and was identified as 3-ethyl-2,5-dimethylpyrazine, which is also used by *Myrmica* species (Attygalle et al., 1985, 1986). There is, on average, 7 ng of the substance in the poison reservoir of each worker (Attygalle et al., 1986). The contents of *Manica rubida* mandibular glands have also been chemically studied (Bestmann et al., 1987), and the ethological activities of the most abundant compounds determined (M.C. Cammaerts et al., 1988). The major substance, manicone, (4*E*)-4,6-dimethyl-4-octen-3-one, is attractive and a locostimulant. It is also the principal constituent of the mandibular glands of *M. mutica* and *M. bradleyi* (Fales et al., 1972), two species of the Neartic regions (Wheeler and Wheeler, 1970). This substance is not present in the mandibular glands of *Myrmica* species.

Manica rubida generally forms large colonies. Behavioral studies, performed essentially in the field, verified the absence of any recruitment in the species, and provided information about nest topology, nestmate recognition, territorial marking and homing (R. Cammaerts and Cammaerts, 1987).

In order to understand how *M. rubida* can survive or be dominant in its appropriate biotope, and in order to extend our pheromonal knowledge of the species, we have now studied its Dufour gland contents both chemically and ethologically. We have compared the Dufour gland contents with that of *Myrmica rubra*, and observed cross-reactions of these two species towards the substances from each other's glands.

METHODS AND MATERIALS

Collection of Ants. Fragments of five nests of *M. rubida* were collected in France, at St André-en-Vivarais (Ardèche, September 1985), and at the Pas de Peyrol, near the Puy Mary (Cantal, August 1986 and September 1987). Nests of *M. rubra* were collected in Belgium. All these colonies were maintained in artificial nests made from plastic boxes partly filled with regularly humidified plaster of Paris and provided with an exit hole for the ants (see R. Cammaerts and Cammaerts, 1987, Figure 4). The artificial nests were placed in polyethylene basins (59 × 38 × 5 cm or 68 × 38 × 7 cm) serving as foraging areas, on which food (dead cockroaches, sugared water) was provided twice a week. Stimuli (sources of pheromones) could be presented to workers on their artificial foraging area.

Another sample of *Manica rubida* from Ochotnica Górna, in the Gorce mountains of Poland was provided dead in dichloromethane and was used only for confirmation of chemical studies.

Chemical Methods. The Dufour glands were dissected under water from freshly killed workers and sealed individually in glass capillary tubes and by that means injected onto the gas chromatograph by the method of Morgan and Wadhams (1972a) without the intervention of solvent. The tubes were kept in the injection port for 2 min at various temperatures from 130°C to 160°C before crushing them. Analysis was carried out on a fused silica capillary column (25 m × 0.32 mm) coated with OV-1 silicone of 0.4 μm film thickness in a Hewlett Packard 5890 gas chromatograph. The carrier gas was helium at 4 psi column head pressure (a flow rate of approx. 1 ml/min). The oven temperature was initially 30°C for 2 min and then increased at a rate of 4°C/min to 270°C. The column effluent was passed straight into the mass spectrometer, a Hewlett Packard 5970B Mass Selective Detector with HP59970C ChemStation data processor. The Mass Selective Detector was set to monitor m/z 35–350, giving a rate of 1.5 scans/sec using 70 eV ionization.

Samples of the dichloromethane extract of the Polish ants were injected (1 μl) onto the same system, using the same conditions, but using a microliter syringe and an inlet splitter with split ratio of 1 : 30 (column–waste).

The very volatile components of the Dufour gland were separated and analyzed by gas chromatography using a Chrompack PLOT fused silica capillary column (10 m × 0.32 mm, Chrompack Ltd., London) coated with PoraPLOT Q (10 μm) in a Carlo Erba (Milan) Fractovap 4160 series gas chromatograph with FID and a Shimadzu Chromatopac C-R3A data processor (Dyson Instruments, Hetton, U.K.). Individually sealed Dufour glands were injected as above. The oven temperature was initially 50°C, then increased at 20°C/min to 150°C. Retention times and peak areas were determined using aqueous solutions of pure compounds of concentrations 5–10 ng/μl.

Ethological Methods. Dufour glands of *M. rubida* or *M. rubra* were dissected in tap water under a binocular microscope and placed on pieces of filter paper (Whatman No. 1, 1 cm^2). Each gland was then presented to the ants, on the piece of paper, in the foraging areas.

Three measures of ethological responses were observed and quantified: the workers' orientation towards the stimulus, their linear speed, and their angular speed in its vicinity. The procedure used is detailed in M.C. Cammaerts et al. (1988). A total of 20 individual workers were observed to calculate the values for the linear and angular speed and 30 individuals for the orientation in each experiment. This furnished a distribution of 20 or 30 experimental values. Each distribution was characterized by its median (and quartiles), and was compared, by means of nonparametric χ^2 tests, with results from other tests or those from control experiments obtained by using untreated pieces of paper.

RESULTS

Chemical Analysis. The amount of very volatile components of the Dufour gland secretion, analyzed on the PoraPLOT Q column, varied in quantity from sample to sample, but the pattern of substances was similar in different individuals. An example of a gland rich in these low-mass, volatile substances is shown in Fig. 1. Peaks corresponding to acetaldehyde (ethanal), ethanol, acetone, 1-propanol, isobutyraldehyde (methylpropanal), butenone, butanone, and 2-butanol are clearly seen. The acetaldehyde and ethanol peaks each represent 4 ng, the acetone peak, 70 ng, but the average amount of acetone was 20 ng. These substances were all identified by comparison of their retention times with authentic specimens. Analysis of *Myrmica* glands on the same column gave a mixture of the same substances but with acetaldehyde or acetone the major component.

Samples of Dufour glands from *M. rubida* workers collected in 1985 and 1987 as well as the preserved sample from Poland all gave similar results for the less volatile components on gas chromatography–mass spectrometry. A typical result for a single gland is given in Figure 2. (*Z*, *E*)-α-Homofarnesene

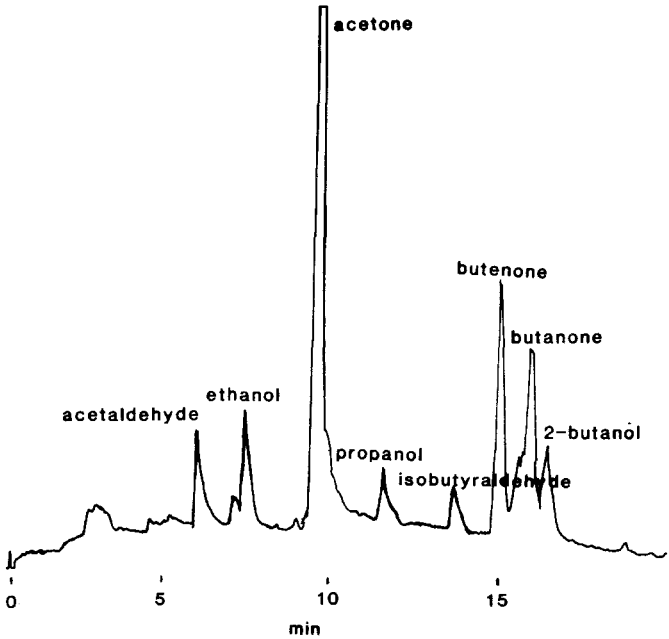

FIG. 1. Gas chromatogram of a single Dufour gland of a worker of *M. rubida*, rich in the volatile components, chromatographed on a PoraPLOT Q column. The small, incompletely resolved peak at 15.7 min is due to butanal or a butenol.

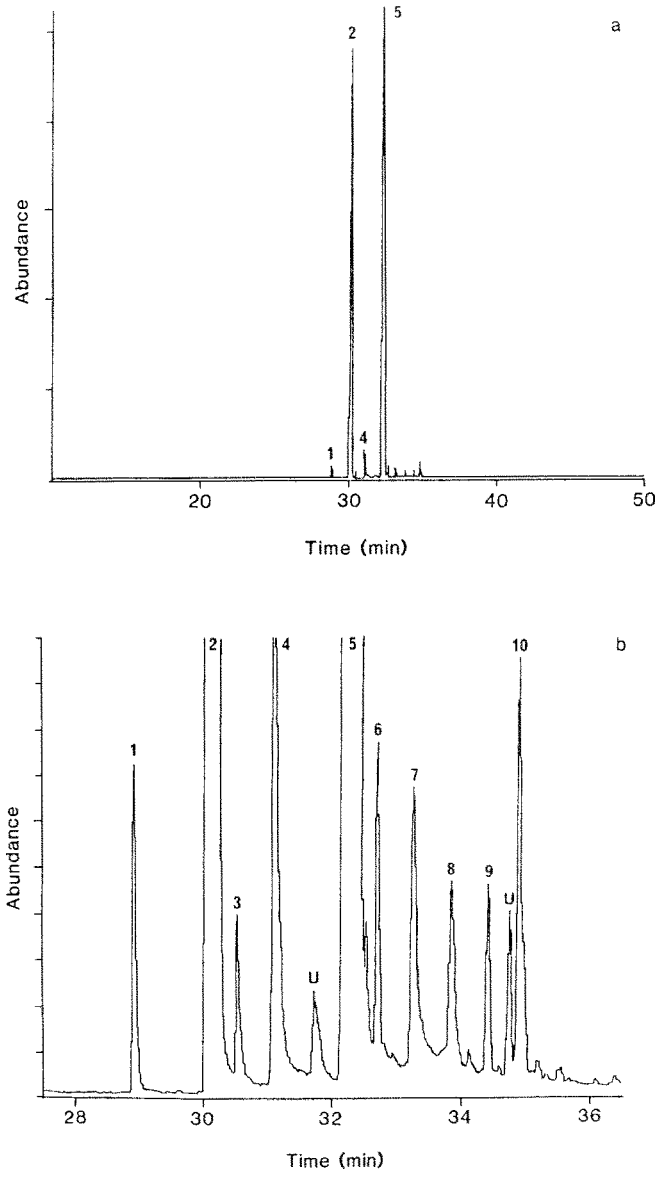

FIG. 2. Gas chromatogram of the contents of the Dufour gland of one worker of *M. rubida*, (a) showing the two main components, (*Z*, *E*)-α-farnesene and (*Z*, *E*)-α-homofarnesene, and absence of other major components. (b) Expansion of the region 28–36 min, to show the minor components. The numbers correspond to those in Table 1. U indicates unidentified substances. This gland was selected as one richer in the minor components. The sum of the masses in all the peaks is approximately 1 μg.

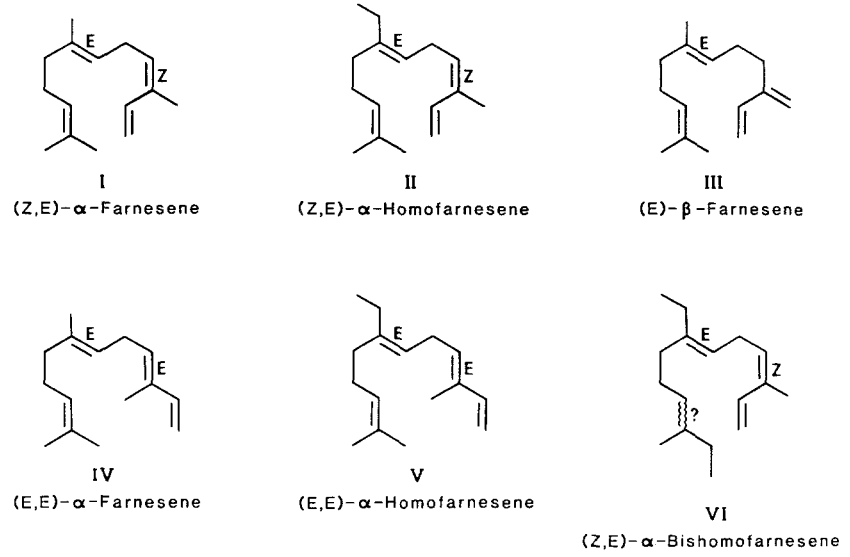

FIG. 3. Structures of farnesene isomers and homologs identified in *Manica rubida* Dufour glands.

(structure II, Figure 3) was in each case the major substance found, with (*Z*, *E*)-α-farnesene (I) next. The ratio of these compounds in individual workers varied from 4:1 to almost equal quantities. In the pooled sample of approximately 100 workers collected in Poland, the ratio was 3:2, which represents an approximate average value for the French sample collected in 1987.

Identification of these two substances was made from their mass spectra and confirmed by comparison of spectra and retention times with the same substances found in *Myrmica* Dufour glands (Attygalle and Morgan, 1984) that have been fully characterized by degradation (Attygalle and Morgan, 1982) and synthesis (Morgan and Thompson, 1985). Much smaller amounts of other farnesene isomers and homologs were also found. These are seen in Figure 2b and listed in Table 1, together with the more diagnostic ions in their mass spectra. (*E*)-β-Farnesene (III) and (*E*, *E*)-α-farnesene (IV) are well characterized (Anet, 1970) and synthetic samples, prepared by dehydration of nerolidol (Morgan et al., 1979) were available for comparison. (*E*, *E*)-α-Homofarnesene (V) was identified by its retention time relative to (*Z*, *E*)-α-homofarnesene and because its mass spectrum closely resembled that of (*E*, *E*)-α-farnesene with the higher mass ions displaced by +14 mass units. (*Z*, *E*)-α-Bishomofarnesene (VI) is the same as the *Myrmica* substance (Morgan and Wadhams, 1972b) confirmed by degradation (Attygalle and Morgan, 1982). Another still smaller peak, identi-

TABLE 1. SUBSTANCES FOUND IN DUFOUR GLAND OF *Manica rubida* WORKERS IN ORDER OF GC ELUTION FROM NONPOLAR COLUMN

No.[a]	Name	Retention index		Mass spectrum *m/z* (with intensities)
1	(*E*)-β-Farnesene (III)	1443	$C_{15}H_{24}$	M^+204(1), 189(1), 161(7), 133(18), 123(1), 120(12), 119(6), 107(6) (105) (7), 93(50), 79(25), 69(98), 55(20), 41(100)
2	(*Z*,*E*)-α-Farnesene (I)	1482	$C_{15}H_{24}$	M^+204(0.3), 189(1), 161(3), 133(3), 123(3), 120(9), 119(61), 107(30), 105(23), 93(85), 79(38), 69(43), 55(44), 41(100)
3	(*E*,*E*)-α-Farnesene (IV)	1492	$C_{15}H_{24}$	M^+204(−), 189(−), 161(3), 135(6), 133(4), 123(18), 120(6), 119(30), 107(39), 93(85), 79(39), 69(47), 55(46), 41(100)
4	(*E*)-β-Homofarnesene-2	1511	$C_{16}H_{26}$	M^+218(0.8), 203(0.4), 189(1), 175(4), 147(10), 134(9), 123(4), 119(7), 107(11), 93(43), 79(24), 69(86), 55(18), 41(100)
5	(*Z*,*E*)-α-Homofarnesene (II)	1554	$C_{16}H_{26}$	M^+218(0.2), 203(0.3), 189(3), 175(1), 147(2), 133(17), 121(7), 119(7), 107(21), 105(30), 93(65), 79(33), 69(47), 55(32), 41(100)
6	(*E*,*E*)-α-Homofarnesene (V)	1564	$C_{16}H_{26}$	M^+218(1), 189(1), 175(2), 149(6), 147(3), 137(20), 133(10), 121(12), 119(10), 107(36), 105(26), 93(72), 79(43), 69(53), 55(37), 41(100)
7	(*Z*)-β-Bishomofarnesene (?)	1583	$C_{17}H_{28}$	M^+232(−), 219(0.1), 205(0.3), 191(0.5), 151(4), 135(3), 121(8), 119(3), 107(11), 95(26), 83(24), 69(100), 55(36), 41(83)

TABLE 1. Continued

No.[a]	Name	Retention index		Mass spectrum *m/z* (with intensities)
8	(*E*)-β-Bishomofarnesene (?)	1605	$C_{17}H_{28}$	M^+232(0.3), 205(0.3), 189(0.5), 161(1), 151(4), 149(3), 147(2), 145(1), 137(4), 123(5), 121(7), 107(11), 95(27), 83(24), 79(14), 69(100), 55(38), 41(81)
9	Bishomofarnesene of unknown structure, same compound as found in *Formica lemani* (Ali et al., 1987)	1622	$C_{17}H_{28}$	M^+232(−), 203(3), 163(2), 161(2), 147(15), 119(23), 107(39), 93(28), 91(27), 79(61), 69(55), 55(30), 41(100)
10	(*Z*,*E*)-α-Bishomofarnesene (VI)	1638	$C_{17}H_{28}$	M^+232(−), 203(3), 175(2), 149(3), 147(3), 133(21), 119(10), 107(26), 105(40), 93(75), 79(40), 69(24), 55(100), 41(74)

[a] Refers to Figure 2.

fied in only some samples, was the bishomofarnesene found in *Formica lemani* (Ali et al., 1987). The (*E*)-β-homofarnesene found here is not the same as that identified in *Harpagoxenus sublaevis* (Ollett et al., 1987). The latter has a quite different mass spectrum with a strong ion at *m/z* 133 (compare with Table 1). The *Manica* substance is therefore called (*E*)-β-homofarnesene-2 until these structures are solved in full. Small quantities of two further bishomofarnesenes were identifiable in some samples (less than 0.5% of the total in the gland).

Behavioral Studies. Freshly isolated Dufour glands of *M. rubida* and of *M. rubra* were presented to workers of *M. rubida*. For comparative purpose, Dufour glands of *M. rubida* were also presented to workers of *M. rubra*. In each case, the locomotion reactions of workers were observed and quantified. The numerical results obtained are reported in Table 2.

They show that *M. rubida* workers do not orient themselves either towards their own or towards *M. rubra* Dufour glands. However, *M. rubra* workers are attracted towards *M. rubida* Dufour gland contents. We know *M. rubra* workers are attracted to their own Dufour gland contents because of a few extremely volatile compounds, chiefly acetaldehyde (Cammaerts-Tricot et al., 1976).

TABLE 2. LOCOMOTION REACTIONS OF *Manica rubida* AND *Myrmica rubra* WORKERS STMULATED BY ANTS' DUFOUR GLANDS[a]

Tested species and presented stimuli	O (ang. deg.)	*P*	V (mm/sec)	*P*	S (ang. deg./cm)	*P*
Manica rubida						
Control	92 (57–105)		18.0 (15.1–21.4)		85 (60–108)	
M. rubida Dufour gland	92 (78–106)	NS	38.0 (32.0–45.6)	$P < 0.001$	92 (80–105)	NS
M. rubra Dufour gland	87 (70–99)	NS	33.0 (27.0–36.0)	$P < 0.001$	116 (102–140)	$P < 0.01$
Myrmica rubra						
Control	105 (70–119)		11.0 (10.0–12.0)		142 (120–158)	
M. rubida Dufour gland	55 (45–67)	$P < 0.001$	30.8 (26.8–34.4)	$P < 0.001$	110 (97–120)	$P < 0.01$

[a]Each time the ant's orientations (=O), linear speed (=V) and angular speed (=S) were quantified, as detailed in Cammaerts-Tricot et al. (1976), by 20 or 30 individual values. The table gives the median (and the quartiles) of the distributions obtained as well as results of non-parametric χ^2 tests between control and experiments. P = level of probability; NS = non significant difference for $P = 0.05$.

These very volatile substances also occur in *M. rubida* Dufour glands, but the workers of that species do not respond to them.

Myrmica rubra workers seem to react positively to *M. rubida* Dufour glands only during the first 1–2 min following the isolation of the gland. This time is somewhat shorter than that found when presenting *M. rubra* Dufour glands to workers of this species. This can be explained by the relatively small amount of acetaldehyde produced by *M. rubida* Dufour glands.

From Table 2 it appears that *M. rubida* workers exhibit a very large increase of linear speed, and almost no change of angular speed in the presence of their own Dufour gland contents. When stimulated by *M. rubra* Dufour gland contents, workers of *M. rubida* exhibit a similar large increase of linear speed and a small increase of angular speed. The reactions to the two glands are thus similar, but not identical.

When perceiving *M. rubida* Dufour gland contents, workers of *M. rubra* present a very large increase of linear speed, and a small decrease of angular speed (Table 2). We know from earlier studies on *M. rubra* Dufour glands that its own gland contents induce a rather large increase of linear speed and an increase of angular speed (Table 3). Therefore, the locomotion reactions produced in *M. rubra* workers, by *M. rubra* and *M. rubida* Dufour glands, are once again similar but not identical.

In short (Table 3), a Dufour gland of *M. rubida* releases a larger positive orthokinesis (and consequently a smaller positive klinokinesis) than a Dufour gland of *M. rubra*, and this is independent of the different locomotion characteristics of the two species studied. It was also noted during observation of the ants, although no quantitative data are given, that an *M. rubida* Dufour gland remained ethologically active for a shorter time than an *M. rubra* Dufour gland.

TABLE 3. CROSS-ACTIVITY OF *Manica rubida* AND *Myrmica rubra* DUFOUR GLANDS

	Glands from					
	M. rubida		*M. rubra*		Means for each species	
Tested species	V	S	V	S		
M. rubida	38	92	33	116	35.5	105
M. rubra	31	110	20	180	25.5	145
Means for each gland	34.5	101	26.5	148		

[a] The ethological activities recorded are the ant's linear (=V, mm/sec) and angular (=S, ang.deg./cm) speed. The values given here are the medians of distributions of 20 experimental values, obtained in the present study (see Table 2) and in previous ones (e.g., Morgan et al., 1977).

DISCUSSION

Chemical Results. Wadhams (1972) has shown from electroantennographic studies that *Myrmica rubra* workers could detect some very volatile components in their Dufour gland. Cammaerts-Tricot et al. (1976) showed that this very volatile and, therefore, short-acting component, elicited characteristic behavioral movements from workers. The mixture contained a number of alcohols, aldehydes and ketones of one to four carbon atoms, with acetaldehyde and acetone most abundant. The cross-activity studies of behavior in the present work indicated that we should look for such volatile components in *M. rubida*. We have done this using gas chromatography, but for the first time with a capillary column coated with a 10-μm layer of a copolymer of styrene and divinylbenzene, which is the same as the porous polymer (Porapak Q) used in the packed columns in the earlier work (Morgan and Tyler, 1977). A similar mixture of volatile substances was found, with acetone the most abundant (Figure 1).

The less volatile component of the Dufour gland secretion is one of the simplest patterns of Dufour gland substances we have encountered, with two components making up 98% of the total secretion. However, seven other substances, all sesquiterpene hydrocarbons, comprise the further 2%. Another three substances, representing together less than 0.1% of the total, are also sesquiterpenes but are of unknown structure. No linear alkanes or alkenes were detected. It was surprising to find (*E*, *E*)-α- and (*E*)-β- isomers present. These are the most stable isomers, and it was suspected that they might have been formed by isomerization, which occurs readily above 140°C (Anet, 1970). Therefore, a series of injections of single glands was made varying the injection temperature from 130 to 160°C, but there was no decrease in the amounts of these isomers at the lower temperature, and so it is concluded they are true components in the gland. Using a temperature of 140°C for other species containing (*Z*, *E*)-α-farnesene, we have found no trace of *E*, *E* isomers. Such a rich mixture of sesquiterpene hydrocarbons has not been encountered before in ant Dufour glands.

Manica is regarded as a close relative of the genus *Myrmica*, indeed *M. rubida* was once included in *Myrmica* (Weber, 1947). It is interesting, therefore, in assessing these experiments to make comparison with our earlier work on *Myrmica*.

In a chemical sense, the substances present in the Dufour gland are superficially similar. Of some 13 species of *Myrmica* ants so far studied (Attygalle et al., 1983; Jackson et al., 1989), all have some farnesene, homofarnesene, and bishomofarnesene in them; in some, these are the major components, but we almost always find them mixed with some linear hydrocarbons in *Myrmica*, and we do not usually find the (*E*)-β- and (*E*, *E*)-α- isomers and corresponding homologs.

Furthermore the mixture of undiluted farnesenes and homologs, as found in *M. rubida*, is much less stable than when diluted with alkanes (Morgan et al., 1989) as they are in *Myrmica*. The undiluted mixture reacts with atmospheric oxygen more rapidly, giving a complex mixture of oxidized and polymerized products (cf. Murray, 1969; Sal'kova et al., 1975). Any pheromone message contained in the *Manica* substances will be shorter-acting than in other species.

Behavioral Observations. From our ethological observations, we first deduced that very volatile substances were likely to be present in *M. rubida* Dufour glands, and this was confirmed by our chemical analysis. The lack of attractancy of the Dufour gland for *M. rubida* workers is consistent with the absence of "group recruitment" in this species (M.C. Cammaerts and Cammaerts, 1985; R. Cammaerts and Cammaerts, 1985), in contrast to the behavior in *Myrmica rubra*, for example.

Second, we observed, in the present work, that the displacement of *Manica rubida* workers near isolated Dufour glands is very similar to that seen on marked areas in natural and in experimental environments (R. Cammaerts and Cammaerts, 1987). Our earlier experiments have demonstrated that this secretion is used for marking territories around nest entrances (R. Cammaerts and Cammaerts, 1987). In *Myrmica rubra* also, the less volatile part of the Dufour gland secretion is used for marking territories (M.C. Cammaerts et al., 1977).

Third, *Manica rubida* and *Myrmica rubra* both react, but somewhat differently, to the less volatile portion of their own and each other's Dufour glands. Both species contain quantities of (*Z*, *E*)-α-farnesene and (*Z*, *E*)-α-homofarnesene, but each contains other components that are not common to both species. We have shown elsewhere (M.C. Cammaerts and Morgan, 1989) that the odor detected by the ants diffuses slowly from the gland.

Fourth, we observed that *M. rubida* Dufour glands remained ethologically active for a shorter time than those of *M. rubra*. This may be because the mixture of pure farnesenes in *M. rubida* will decompose more rapidly when exposed to the oxygen of the air than will the same substances diluted with linear hydrocarbons in *M. rubra* secretion (Morgan et al., 1989).

It may be that *Manica rubida* is able to form large and vigorous colonies, although recruiting no nestmates, because it is highly polygynous, because workers continually forage, marking the territory all around the nest entrances and so discouraging the intrusion of other ant species, and because collecting ants appear to be responding to some stimulus (need for food, liquid, nest material) by returning to the same nest entrance from which they last emerged (R. Cammaerts and Cammaerts, 1987). We believe this interesting species will repay further behavioral studies.

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HONEYBEE RESPONSE TO QUEEN MANDIBULAR PHEROMONE IN LABORATORY BIOASSAYS

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Abstract—Quantitative laboratory biosassays measuring short-range attraction of worker honeybees to pheromone-treated pipets or glass pseudo-queens are described. Each replicate involves only 15 workers, allowing a single colony to provide sufficient individuals for a complete study as well as for between-colony comparisons. The more sensitive pseudo-queen bioassay provides a measurable response at levels of queen mandibular extract of 10^{-7} than that of an average individual mated queen. Formation and maintenance of the pseudo-queen's retinue, as well as more detailed behavior, can be evaluated by following the actions of individual workers replayed on video tape. Using this sensitive bioassay, the response of workers to queen mandibular pheromone has been shown to be sensitive to daily variation and colony source of the workers, but independent of worker age.

Key Words—*Apis mellifera* L., honeybee queen mandibular pheromone, retinue behavior, attraction bioassays, Hymenoptera, Apidae, honeybee pheromone, (*E*)-9-keto-2-decenoic acid, (*R*,*E*)-9-hydroxy-2-decenoic acid.

INTRODUCTION

Many activities of worker honeybees, *Apis mellifera* L., are regulated by a complex of semiochemicals, the full chemical identities and behavioral effects of which are, in most instances, unknown (Free, 1987; Winston, 1987; Gary, 1974). Identification of pheromones requires a simple, but discriminating, quantitative bioassay that highlights the behavior being investigated. One such behavior is the formation of a retinue or royal court around the reproductive

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queen honeybee (Velthius, 1985). Workers form a dynamic circle around her, touching her with their antennae and licking her. Many experiments have indicated that the behavior is initiated and maintained by chemicals from the mandibular glands in the queen's head (Gary, 1961). Although many compounds have been identified from this source, no single component was capable of reproducing retinue behavior.

Several worker retinue bioassays have been described that can be carried out both in the colony (Simpson, 1979) and in the laboratory (Pain, 1966; Pham et al., 1982, Velthuis, 1972). Simpson's (1979) study was of special interest as he initiated chemical fractionation studies and assayed the fractions within the colony. Replication necessitated frequent manipulation of several colonies with differing characteristics. Laboratory bioassays circumvented these problems by utilizing workers from a single colony tested under controlled conditions. However, the low sensitivity of these bioassays demanded large initial quantities of queen extract and made fractionation and reconstitution of stimuli without loss of potential volatile constituents cumbersome.

We describe two quantitive laboratory bioassays that utilize workers from a single colony and are readily replicated. Their utility has been established in the determination of the composition of the mandibular pheromone of the mated queen honeybee (Slessor et al., 1988). We also describe the application of the bioassays in determining the response of worker honeybees of differing ages and origins to retinue pheromone.

METHODS AND MATERIALS

Preparation of Bioassay Cages and Chemical Stimuli. Worker bees were removed at random from the upper recesses of a 2-Langstroth deep-super colony between 10:00 and 10:30 AM, lightly narcotized with carbon dioxide, apportioned in groups of 15 into bioassay cages or arenas, and held at room temperature for 3–5 hr with access to 50% aqueous sugar syrup. Stimuli were presented at known concentrations, 1 Qeq representing the average content of the mandibular glands of one honeybee queen. This pheromone composition was established by analysis of a mandibular gland extract obtained from several mated queens (Slessor et al., 1988), and comprised, on average, 150 μg of (*E*)-9-keto-2-decenoic acid, 55 μg of 67% (*R*,*E*)-(−)-9-hydroxy-2-decenoic acid, 13 μg of methyl *p*-hydroxybenzoate, and 1.5 μg of 4-hydroxy-3-methoxyphenylethanol per queen. Fresh stimuli were prepared daily in advance, coded so that the observer did not know the order or identity of the stimuli, and placed randomly on drying racks. Each group of workers was used only once, killed by freezing, and the containers washed in hot water and air dried. Replicates consisted of fresh treatments of identical stimuli to untested bees on the same or

subsequent bioassay days. Both laboratory bioassays were readily performed through the year.

Micropipet Bioassay. The wooden cages used for micropipet bioassays measured 10 × 10 × 6.5 cm, with the back large side covered with screening and the front with a sliding Plexiglas door. The stimuli consisted of two micropipets, one containing a solvent control and the other an extract, fraction, or synthetic compound placed against the screen at the bottom of the cage. A single stimulus presented within a cage of worker honeybees previously was used for an attraction bioassay (Pham et al., 1982). Stimuli were prepared by drawing a pheromone solution in methanol into the micropipet and allowing the solvent to evaporate while the pipets were maintained at an angle with the aperture directed downward, thereby depositing any residuals at the tip.

A response was recorded when any worker contacted either pipet by reaching through the screen with either its antennae, mouth parts, or tarsi during the 5-min observation period. Responses were summed as a measure of the activity for each assay.

Pseudo-Queen Biosassay. Bioassay arenas were prepared from disposable plastic Petri dishes (15 × 2 cm). One circular hole (1.5 cm diam.) was cut into the top and covered with screening, through which the bees were fed. Another hole (1 cm diam.) was cut in the side for introducing a glass pseudo-queen. Pseudo-queens are prepared from a 7-cm length of a 1-ml Pasteur pipet (2 cm of the top and 5 cm of the narrowed bottom), cut and sealed at both ends. A small indentation suitable for receiving 10 μl of test solution was formed in the larger "head" end. On evaporation of the solvent, the pseudo-queens were stored for 2–3 hr in a refrigerator until 2–3 min before a bioassay.

Upon introduction of the pseudo-queen stimulus, activity within the dish was recorded on video tape for 5 min. At 30-sec intervals the number of workers contacting the stimulus area of the pseudo-queen and those within an "active ellipse," i.e., within 1 worker length (1.5 cm) of it, were recorded. The contact and ellipse counts were summed separately for each replicate.

Persistence. Early observations using the micropipet technique had shown that some worker honeybees that found the lure would not vacate their position and allow others to contact it. Such activity prevented the positive response of other workers and illuminated the persistent behavior of individual workers to remain in contact with the stimulus. With the more accessible pseudo-queen lure, a measure of this persistence was established by review of the video tapes. The time spent by each bee that had entered the ellipse within the first 2 min of the trial was measured to a maximum of 3 min.

Age Study. Frames containing emerging brood were removed from a single colony and placed in an incubator at 33°C and 70% relative humidity. Workers that emerged overnight were marked with an enamel paint dot on the thorax

and returned to the parent colony. This procedure was repeated every second day for 30 days (July 20–August 19, 1987), using a different color paint, and resulted in marked bees comprising 15 age groups between 1 and 29 days old.

Pseudo-queen bioassays were then conducted as previously described, but with workers of known ages. Test bees were chosen so that one member of each age group was in each arena, i.e., 15 workers per arena. Fifteen stimulus replicates and one blank were tested on each of the two test days. In this way, 30 individuals from each age class were tested. Glass pseudo-queen stimulus containing 10^{-3} Qeq of synthetic retinue pheromone were used. Workers that remained within the ellipse for >1 min were scored and their age recorded.

Colony Variation. The responses of workers from 12 colonies of *A. mellifera ligustica*, eight headed by domestic queens (Southern Interior Apiaries, Keremeos, B.C.) and four headed by imported queens (Haines Bee Breeders, Kaitaia, New Zealand) were compared in the pseudo-queen bioassay with 10^{-2} Qeq stimuli of synthetic retinue pheromone. A replicate consisted of one group from each of the 12 colonies. There were two groups of 10 replicates tested on February 2–24 and May 10–June 1, 1988. At the conclusion of the May experiment, the best- and worst-responding colonies were chosen for a 16-replicate experiment to evaluate their response at doses of 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} Qeq.

RESULTS AND DISCUSSION

The response of workers to honeybee queen mandibular synthetic pheromone was dose dependent (Figure 1). In the micropipet bioassay, the response to stimuli of 10^{-1} and 10^{-2} Qeq was significantly different from the response to solvent control (Tukey's multiple-range test, $P < 0.05$), a slightly more sensitive response than in the cage bioassay described by Pham et al. (1982). The exclusive position established by persistent workers at the pipet and the relatively small size of the lure in relation to the cage volume, which frequently resulted in the workers not coming near the stimulus, reduced the sensitivity of the bioassay. Nevertheless, the pipet bioassay was used to establish the involvement of several relatively polar molecules that are components of the queen mandibular pheromone that elicits retinue behavior (Slessor et al., 1988). The recognition volume of this pheromone signal must be quite small compared to many other insect pheromones, e.g., lepidopteran sex pheromones, since the full blend is necessary for complete perception and several of the components have very low volatilities. The direct contact of the workers with the pheromonal source observed in both bioassays implies a physical transfer of semiochemical rather than perception of an airborne signal. Queen presence studies, as measured by the behavior of workers kept at noncontact distances, support this concept (Free, 1987; Winston, 1987).

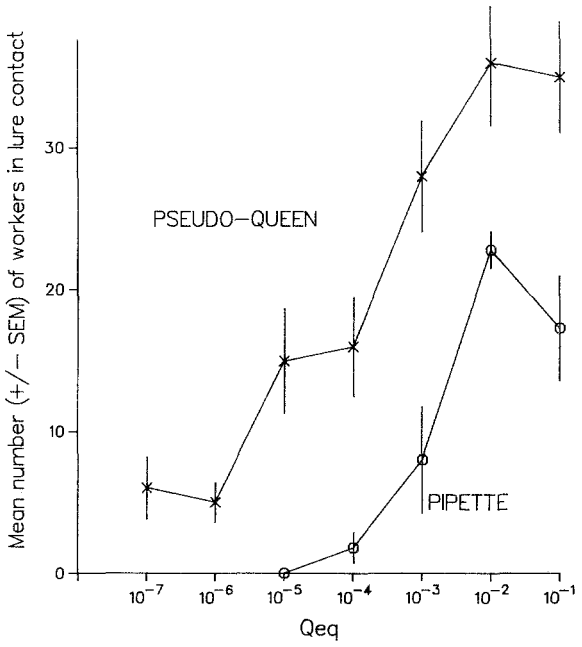

FIG. 1. Responses of worker honeybees to stimuli containing various quantities of synthetic mandibular pheromone in the two types of bioassay.

The pseudo-queen bioassay was considerably more sensitive to worker response than the micropipet technique. Workers oriented head-first to the glass pseudo-queen, touching it with their antennae and/or tongue and tarsi. Positive responses were obtained at 10^{-7} Qeq of synthetic pheromone, demonstrating the sensitivity of worker honeybees to extremely small quantities of queen mandibular pheromone that signals the presence of a queen within a strong and vigorous colony. There was no greater sensitivity in contact data over ellipse data at a given extract concentration.

Bimodal behavior patterns were observed in the persistence of individual workers to both queen mandibular extract and synthetic queen mandibular substances (Figure 2). Typical retinue behavior was exhibited by 25–30% of the bees, including prolonged antennation and licking of the lure, occasional turning away to groom their mouth parts or to antennate with other bees, but no movement from the active ellipse. Longer visits (>2.5 min) were clearly a response to chemical stimuli while short visits (<30 sec) appeared to be random or exploratory. Natural and synthetic queen mandibular retinue pheromones were different from solvent (Kolmogrov-Smirnov, two-tailed test, Kleinbaum and Kupper, 1978; Zar, 1984, $P < 0.001$) but not significantly different from each other ($P > 0.10$). The percentage of worker bees responding and their

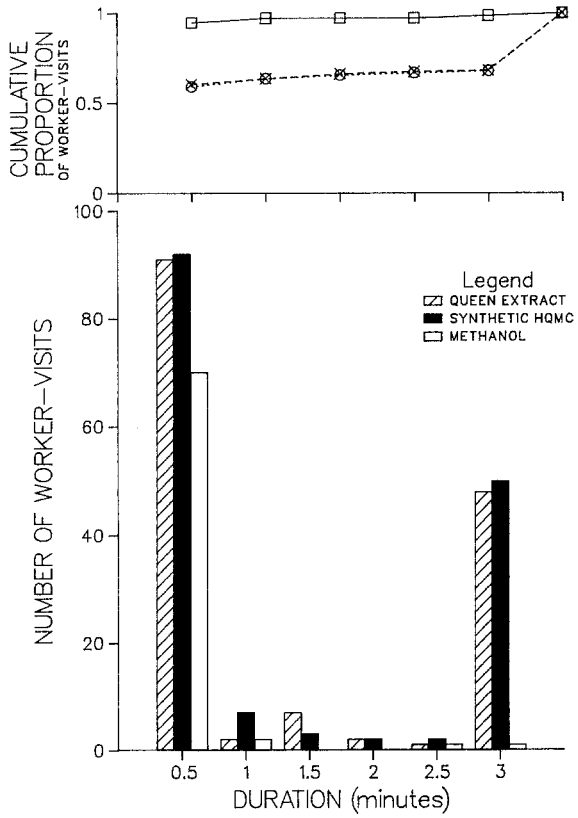

FIG. 2. Persistence of worker honeybees in the pseudo-queen bioassay to natural and synthetic mandibular pheromone, as well as methanol solvent. Persistence is defined as the time spent in any visit by a worker bee entering the active ellipse during the first 2 min of a bioassay. Run at 10^{-3} Qeq ($N = 9$). Symbols for cumulative response curves are: X = queen, ○ = synthetic mandibular extract, and □ = methanol solvent.

persistence were greater than those of the messenger bees described by Seeley (1979); however, our experimental bees were queen- and task-deprived and had been treated with CO_2, any one of which might have caused an enhanced response to the queen's semiochemicals.

No age dependence was observed in response of worker bees to pseudo-queens treated with retinue pheromone (Figure 3). Of the 450 bees tested, 42 remained within the ellipse >1 min. The mean age of the responders was 14.0 days, within the range found for queen attendants in observation hives [5.5 days (Seeley, 1979), 10.7 days (Allen, 1960), and 17.1 days (Winston and Punnett, 1982)]. In the attraction bioassay (Pham et al., 1982), a greater response was

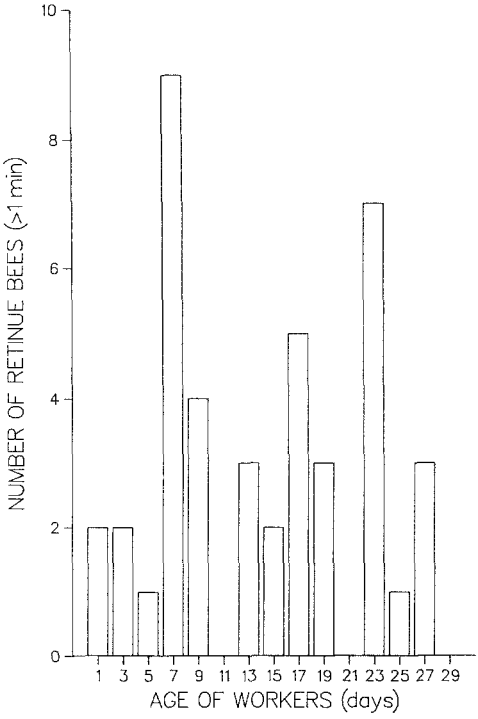

FIG. 3. Ages of worker bees remaining >1 min in the active ellipse during a 5-min pseudo-queen bioassay. Run at 10^{-3} Qeq of synthetic mandibular pheromone, (N = 30).

observed with very young 3-day-old workers, suggesting a different behavioral response. The workers observed in the present study were raised in late summer, and, consequently, their development and behavior may not fully match workers raised earlier in the year where life-spans would be greatly reduced. Nevertheless, the lack of age dependence facilitates the use of the pseudo-queen bioassay, since marked age cohorts are not necessary to produce repeatable results.

Significant variation in contact response to 10^{-2} Qeq stimuli of retinue pheromone existed between colonies measured in February and May (Table 1). Workers from colonies headed by queens from New Zealand were less responsive than bees derived from British Columbia queens at both dates. Daily variation in response to stimuli was large and did not appear to be directly correlated with weather conditions, including pressure changes. Workers from the highest and lowest responding colonies exhibited significant differences in response to stimulus concentrations 10^{-5} Qeq and above (t test, $P < 0.05$) (Figure 4).

TABLE 1. MEAN NUMBER OF CONTACTS IN PSEUDO-QUEEN BIOASSAY RELATIVE TO QUEEN ORIGIN AND TIME OF YEAR

Queen origin	*N*	February[a]	May[a]	Differences between dates
British Columbia	8	13.5 ± 1.4a	18.2 ± 1.4a	<0.025
New Zealand	4	2.7 ± 0.5b	8.4 ± 1.7b	<0.001
Mean	12	9.9 ± 1.1	15.2 ± 2.0	

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

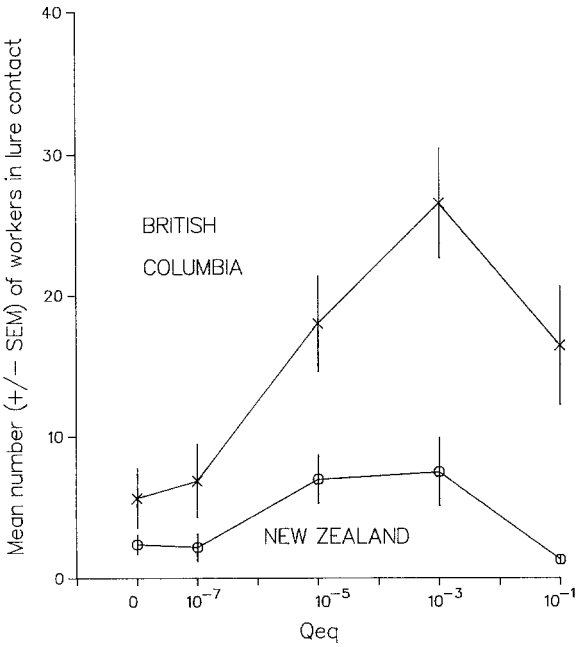

FIG. 4. Contact responses to various stimulus concentrations by worker honeybees from two colonies selected on the basis of the strongest (headed by a queen from British Columbia) and weakest (headed by a queen from New Zealand) response in the pseudo-queen bioassay during June 1988. All concentrations were run with both sets of bees each day for eight days ($N = 16$).

Subsequent analysis of these two queens for their mandibular components, both within the individual glands and over the remainder of the body, showed no significant difference or correlation with the contact response observed for that colony. A weak, but significant correlation of response to the size of the colony population was found ($R^2 = 0.68$, $P < 0.05$), but no significant correlation with the size of the brood area was observed ($P > 0.05$). The correlation of response with colony population may result from a decreased ability of workers in large colonies to distribute queen pheromone. Between-colony variation has implications for the breeding and selection of queens if queen pheromone is correlated with productivity or other queen traits. The lower response to stimuli at 10^{-1} compared to 10^{-3} Qeq indicates that response is diminished when high amounts of synthetic queen mandibular pheromone are presented, at least in the bioassay situation.

CONCLUSIONS

Our facile laboratory bioassays quantitatively evaluate the presence and retinue effect of queen mandibular pheromone on worker honeybees. The simpler pipet bioassay has the advantages of minimal equipment investment and greater probability of detection of more volatile worker attractants. The more complex pseudo-queen bioassay provides a substrate more accessible to worker honey-bees for the presentation of queen pheromones. These bioassays have already proven to be of value in the isolation and identification of the components of the mandibular retinue pheromone of queen honeybees (Slessor et al., 1988). The quantitative aspects of the pseudo-queen bioassay add to its utility. In this study, it was employed to demonstrate the persistent behavior of some workers in the presence of queen retinue pheromone, to demonstrate that there is little or no age variation in the bioassay response of worker honeybees to the mandibular retinue pheromone, and to demonstrate that variations in response occur within a colony on a day-to-day and seasonal basis, and between colonies on the same day.

Acknowledgments—We thank M.H. Wyborn for assistance in maintaining bees and bioassays, R.D. Routledge and S.A. Kolmes for statistical evaluations, G.G. Grant for observational insights, and the Natural Sciences and Engineering Research Council of Canada (Operating Grants A3785, A3881, and A7774, Strategic Grant G0958) and the Wright Institute for financial support.

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SEMIOCHEMICALS OF THE HONEYBEE QUEEN MANDIBULAR GLANDS

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Abstract—The ontogeny of the five queen mandibular gland semiochemicals that initiate and maintain the retinue behavior of worker honeybees was investigated by quantitative splitless capillary gas chromatography. No detectable pheromone is present at the time of eclosion, but decenoic acid levels build up rapidly during the first week of the queen's life. Two aromatic components attain detectable levels later, with the more plentiful methyl *p*-hydroxybenzoate preceding the 4-hydroxy-3-methoxyphenylethanol. Pheromone levels are maximal in mature, mated, laying queens. The ratio of (*R*,*E*)-(−)-9-hydroxy-2-decenoic acid to the (*S*,*E*)-(+) enantiomer increases with the age of the queen. Pheromone levels in queen mandibular glands are largely unaffected by queen banking, restraint with workers in mailing cages, and limited storage on dry ice. All major body parts of typical queens, especially the head and legs, have sufficient mandibular exudate to be highly attractive to worker bees.

Key Words—Hymenoptera, Apidae, *Apis mellifera* L., honeybee queen mandibular semiochemicals, retinue pheromone, splitless capillary gas chromatography, (*E*)-9-keto-2-decenoic acid, methyl *p*-hydroxybenzoate, 4-hydroxy-3-methoxyphenylethanol. (*R*,*E*)-(−)- and (*S*,*E*)-(+)-9-hydroxy-2-decenoic acid.

INTRODUCTION

The importance of semiochemicals for queen recognition and caste maintenance within honeybee colonies, *Apis mellifera* L., has been well documented (Free, 1987; Winston, 1987). The five substances that are secreted from the mandib-

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ular gland of the queen that initiate and maintain retinue behavior are: (*E*)-9-keto-2-decenoic acid (9ODA), (*R*,*E*)-(−)- and (*S*,*E*)-(+)-9-hydroxy-2-decenoic acid (9HDA), methyl *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor et al., 1988). The first recognized queen pheromone, 9ODA (Callow and Johnston, 1960; Barbier and Lederer, 1960), is also attractive to mating drones (Gary, 1962). Only when the complete pheromone complement is present are worker bees attracted and full retinue behavior exhibited. Inside the colony, the queen mandibular pheromone suppresses the formation of emergency queen cells, and, outside the colony, it provides a focal point for swarm clustering (Winston et al., 1989). Thus, the chemical composition of the mandibular glands throughout the life of queen honeybees is crucial to the normal response of workers within that colony to the presence and absence of their queen.

Analysis of 9ODA, as the methyl ester, in queen mandibular glands has demonstrated a fluctuation with time of day and year (Pain and Roger, 1978; Pain et al., 1974). Quantitative analysis of polar organic compounds, such as the components of the queen retinue pheromone, is readily accomplished by their conversion into trimethylsilylated derivatives by bistrimethylsilyltrifluoroacetamide (BSTFA) (Gehrke and Leimer, 1971). Gas chromatography of these derivatives on packed columns provided evidence for the compositional changes that take place in the mandibular glands of false queens (Crewe and Velthuis, 1980). Splitless capillary gas chromatography (SCGC) provided increased resolution and sensitivity for investigations of queen mandibular extracts (Crewe, 1982). SCGC coupled with mass spectroscopy (SCGC-MS) provides a means of establishing the homogeneity of a given peak and consequently its purity. Determination of the chirality of alcohols present in single insects has shown that mandibular 9HDA varies in enantiomeric composition between queens (Slessor et al., 1985).

We describe the chemical composition of the five components of the honeybee queen mandibular pheromone, as measured by SCGC of mandibular gland extracts, during the life of queens of different ages, colony exposure, and backgrounds.

METHODS AND MATERIALS

Sources of Queens. Queens less than 6 months of age were raised in our apiary in May 1987 from California stock (Vaca Valley Apiaries, Vacaville, California, with the exception of unmated, 12-day-old queens (obtained from Southern Interior Apiaries, Keremeos, B.C. in May, 1988). Older queens were originally 1985 California stock that had headed Simon Fraser University colonies for two years and became available due to requeening.

Analyses of Mandibular Gland Components. To a portion of a methanol extract (2–20 μl), prepared from both glands of individual queen bees as described by Slessor et al. (1988), an internal standard containing 0.66 μg of decanoic acid in methanol (2 μl) was added and the sample subjected to reduced pressure until the solvent was just removed. Standards of the components of interest showed no diminution when subjected to this treatment. Trimethylsilylation was accomplished through addition of BSTFA (5 μl) (Sigma Chemical Co., St. Louis, Missouri). The solution was agitated and left at room temperature for 40 min. Redistilled, reagent-grade hexane (100 μl) was added, the solution mixed, and a portion (2–3 μl) injected in splitless mode onto a 30-m DB-1 (J & W Scientific, Inc., Rancho Cordova, California) column on a Hewlett-Packard 5880 gas chromatograph (90°C for 5 min 10°C/min to 200°C, isothermal for 5 min). Integration responses were measured on a flame ionization detector calibrated with known standards for each mandibular component. Determination of the chirality of the 9HDA was done on a portion (5 μl) of the methanol mandibular gland extract (Slessor et al., 1985).

The final unknown component of queen mandibular pheromone was isolated by acetylation and microcolumn silica gel chromatography. The structure was assigned by SCGC-MS and high-resolution ^{1}H nuclear magnetic resonance (NMR) of the resulting diacetate.

Labile and Volatile Constituents. Several studies have suggested that volatile or labile components are involved in retinue response (Butler, 1961; Pain, 1961). To test this hypothesis, a freshly prepared queen mandibular extract was fractionated by high-performance liquid chromatography on a reverse-phase column under conditions separating the primary components into four fractions, including the late-eluting nonpolar constituents. The individual fractions were tested in combination with the five-component synthetic pheromone and together as a reconstituted extract in comparison to unfractionated extract in the pseudo-queen bioassay (Kaminski et al., 1990)

Synthetic Pheromones. The decenoic acids were synthesized according to the methods of Lombardo and Taylor (1978) for 9ODA and Kandil and Slessor (1983) for (*S*)-(+), (*R*)-(−), and (±)-9HDA. HOB and HVA, the latter recrystallized from ethyl acetate: 30–60°C petroleum ether (mp 60–61°C), were obtained from Sigma Chemical Co., St. Louis, Missouri. A queen equivalent (1 Qeq) is arbitrarily defined as 150 μg of 9ODA, 55 μg of 71% (*R*)-(−)-9HDA, 13 μg of HOB, and 1.5 μg of HVA.

Mandibular Gland Compositional Response to Banking and Extreme Cold. To examine the effect of type and duration of queen banking on quantities of queen mandibular pheromone, 2-year-old mated laying queens were restrained in individual queen cages and placed in the top of a two super colony (banking). A portion of the queens was removed after 10 days; some queens were placed

in mailing cages with five workers and sugar candy and kept at room temperature for five days, others were frozen with Dry Ice and kept at −78°C on Dry Ice for seven days, and still others were given both treatments (mailing cages, then Dry Ice). The remaining queens were removed from the colony after 30 days and half were kept on Dry Ice for a further seven days. At the end of each of these treatments, mandibular glands were removed and analyzed for their pheromone composition.

Mandibular Components Present on Queen Body Parts. The amounts of retinue pheromone components present on the body segments of queens that had been kept with five workers in mailing cages for several days were evaluated through analysis of the amounts of 9ODA present in methanol washes of these parts. Seven pooled samples comprising 37 queens were analyzed.

RESULTS AND DISCUSSION

Structural Assignment of HVA. The structural assignment of HVA, a compound new to honeybees, is described for the first time. The isolated diacetate (2–3 μg) provided a SCGC-MS spectrum with a dominant base peak at m/z = 150; as well as 135, 20%; 137, 18%; 210, 8%; 192, 4%, and 252, 2%, reminiscent of the material found by Crewe and Velthuis (1980) in *A. mellifera* queens, and thought to be 4-hydroxy-2-methoxyphenylethanol. When this material was synthesized,[3] it was neither active in bioassays nor did it cochromatograph with the insect-derived material (Slessor et al., unpublished data). The diacetate isolated from five mated, laying queens yielded a proton NMR in CD_2Cl_2 at 400 MHz for 114 hr (97,456 scans), δ = 2.01 (3H, singlet, acetoxy methyl); δ = 2.26 (3H, singlet, aryl acetoxy methyl); δ = 2.92 (2H, triplet, J = 7 Hz, benzylic methylene); δ = 3.08 (3H, singlet, aryl OCH_3); δ = 4.26 (2H, triplet, J = 7 Hz, CH_2O); δ = 6.80 (1H, quartet, J = 2 Hz, 8 Hz, H_6aryl); δ = 6.85 (1H, doublet, J = 2 Hz, H_2aryl); δ = 6.94 (1H, doublet, J = 8 Hz, H_5aryl). A 10,000-scan decoupling of the δ = 2.92 signal eliminated the ~0.5-Hz coupling to the δ = 6.80 and δ = 6.85 aryl hydrogen, confirming these hydrogens as ortho to the ethanol side chain. The chemical shifts of the aryl Hs indicated the substitution as 3-methoxy and 4-hydroxy. Recrystallized HVA was active in the bioassays when incorporated at the appropriate concentration. When silylated, it cochromatographed with insect-derived material on the splitless capillary system; when acetylated, it gave identical retention characteristics and mass spectral fragmentation as the isolated substance.

[3] The reported structural assignments in the synthesis of 4-hydroxy-2-methoxyphenylethanol (Howard et al., 1981) are reversed. See Kaufman and Russey (1965) for the correct structural precursors.

Tests for Volatile or Labile Queen Mandibular Components. Reconstitution of the four fractions of fresh mandibular extract provided an identical worker response to the original extract and synthetic retinue pheromone in the pseudo-queen bioassay at 10^{-3} Qeq (Table 1). Thus, no labile or volatile retinue semiochemical component had been lost in storage or in the fractionation procedure.

Individual Queen Mandibular Glands. Extracts from the mandibular glands of individual queens that had eclosed within the previous 24 hr showed little, if any, semiochemical. Increased levels of 9ODA and 9HDA, and very little HOB, were found in virgin queens six and 12 days after eclosion (Table 2). The presence of significant levels of 9HDA in queens capable of mating and the demonstration of the presence of other active substances involved in the mating process (Gary, 1961) indicate that the 9HDA enantiomers should be further investigated in the attraction of drones and the mating of honey bee queens. Orientation of swarms to a virgin queen is often less effective than to a mated queen. Workers occasionally fail to locate virgins, often forming their main cluster close to but not on a virgin queen. They take longer to find virgin than mated queens and are so frequently restless that swarm clusters may break. The absence of the aromatic mandibular components essential for the full retinue response may underline the observed differences between attraction of workers to virgin and laying queens during swarming (Winston et al., 1989).

Mandibular gland extract from mature laying queens contained significantly enhanced levels of the aromatic constituents (HOB and HVA), compared

TABLE 1. RETINUE RESPONSE BY WORKER HONEYBEES TO GLASS PSEUDO-QUEENS TREATED WITH FRESHLY PREPARED QUEEN MANDIBULAR GLAND EXTRACT, AND FRACTIONS COMBINED WITH SYNTHETIC RETINUE PHEROMONE[a]

Treatment	Number of bees within the active ellipse of the pseudo-queen ($\bar{X} \pm$ SE)[b]
Mandibular gland extract	72.6 ± 10.6 a
Reconstituted, four = fraction extract	53.1 ± 8.0 a
Synthetic, five = component pheromone	78.3 ± 11.1 a
Synthetic pheromone + fraction A	59.9 ± 7.5 a
Synthetic pheromone + fraction B	70.7 ± 8.9 a
Synthetic pheromone + fraction C	83.2 ± 10.2 a
Synthetic pheromone + fraction D	60.9 ± 7.6 a
Methanol solvent	7.8 ± 2.1 b

[a] All stimuli tested at 10^{-3} Qeq, 9 replicates per treatment.
[b] Means followed by the same letter are not significantly different, ANOVA, $P < 0.05$.

TABLE 2. SEMIOCHEMICAL CONTENT OF MANDIBULAR GLAND EXTRACTS FROM HONEYBEE QUEENS OF DIFFERENT AGES, MATING STATUS, AND LAYING EXPERIENCE[a]

		Honeybee queen mandibular semiochemical content (μg)								Chirality of 9HDA %(*R*)-(−)	
		9ODA		9HDA		HOB		HVA			
Queen status	*N*	$\bar{X} \pm$ SE	Range	$\bar{X} \pm$ SE	Range	$\bar{X} \pm$ SE	Range	$\bar{X} \pm$ SE	Range	$\bar{X} \pm$ SE	Range
Eclosed within previous 24 hr	10	0 ± 0 a	0–1.5	0.5 ± 0.1 a	0–1	0 ± 0 ab		0 ± 0 a			
Unmated, in small colonies 6 days	7	161 ± 21 b	100–240	24 ± 3 a	10–33	0.4 ± 0.1 ab	0–0.7	0 ± 0 a		62 ± 3 a	50–72
Unmated, in small colonies 12 days	15	205 ± 22 b	54–360	29 ± 4 a	9–63	0.4 ± 0.1 ab	0.2–0.7	0 ± 0 a		65 ± 3 a	50–72
Mated, laying 1 day	9	244 ± 39 b	53–454	51 ± 9 a	8–98	1.8 ± 0.4 ab	0–3.5	0.07 ± 0.04 a	0–0.3	79 ± 2 b	70–86
5 weeks	10	199 ± 26 b	65–295	147 ± 16 b	96–225	12.9 ± 1.5 bc	5–18	1.3 ± 0.1 ab	0.7–2.2	93 ± 1 d	88–96
2 years old mated, laying	55	265 ± 14 b	63–463	111 ± 7 b	12–238	16.0 ± 1.9 c	0–67	2.3 ± 0.3 b	0–7.7	85 ± 1 c	64–96

[a] Means within columns followed by the same letter are not significantly different at $P < 0.05$ in Tukey's studentized range test for the components and an arcsine transformation Waller-Duncan test, K ratio = 100, for the chirality of 9HDA.

to that in virgin queens. This trend is in keeping with the necessity of fully functioning queens to initiate and maintain retinue behavior. The low levels or absence of aromatic compounds in virgin and newly mated queens, in contrast to laying queens, may explain in part why younger queens are accepted relatively easily by a queenless colony.

Variations in Chirality of 9HDA. The percentage of (*R*)-(−)-9HDA increased significantly when queens were mated and increased further when they had been laying for five or more weeks. Both enantiomers of 9HDA are required for optimal formation and maintenance of the retinue response (Slessor et al., 1988); the (*R*)-(−) enantiomer is most effective in the maintenance of the swarm cluster (Winston et al., 1982). Further implications of the importance of the chirality of 9HDA must await a better understanding of the biosynthetic and metabolic interconversions of the two 9HDA enantiomers and 9ODA.

Although considerable variation in the amounts of pheromone components from individual mature laying queens is observed, a pattern of two- to threefold excess 9ODA to 9HDA, with 1/10 as much HOB and 1/100 as much HVA is common (Figure 1).

For 2-year-old queens, there were no significant differences ($P < 0.05$) in

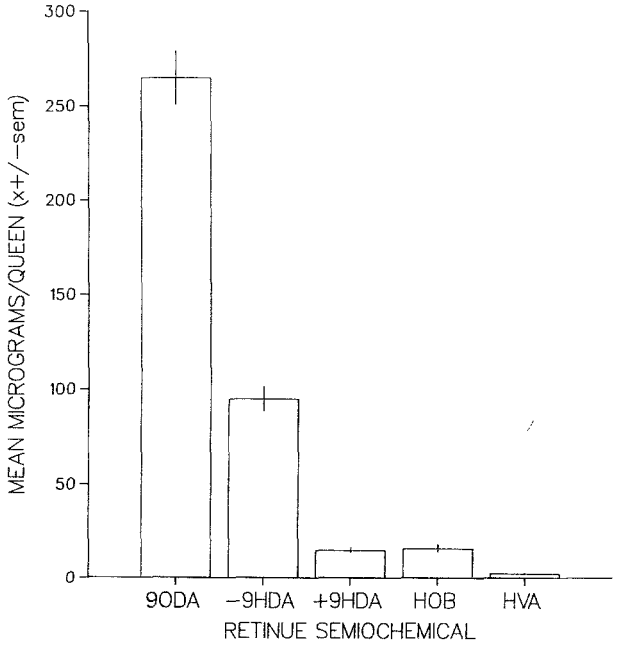

FIG. 1. Average composition of retinue pheromone components in mature, mated laying queens ($N = 55$).

the amounts of the five mandibular pheromone components between mated, laying queens that had been banked ($N = 39$) and control ($N = 16$) queens that had not received banking treatments. Moreover, control queens and those caged with workers and sugar candy in typical queen mailing cages for five days to simulate shipping ($N = 13$) contained similar quantities of the pheromone components. Therefore, all data on 2-year-old queens were pooled (Table 2). We conclude that common treatments of queens in commercial shipping procedures would probably not diminish their capacity to control pheromone-mediated behavior in worker bees. However, the rate of pheromone production could be temporarily altered by banking or similar treatments, and such changes would not be observed by quantitative analysis of stored pheromone in the mandibular glands. Storage of queens ($N = 19$) on Dry Ice (ca. $-78°C$) for seven days resulted in no discernible changes in pheromone amounts or composition.

Determination of queen mandibular pheromone components, other than 9ODA, on queen body segments were often lower than detection limits but, when observed, showed ratios corresponding to their mandibular gland origin. The levels of retinue pheromone found on the head and legs of queens caged with workers for five days were those found to be most attractive to workers in the laboratory pseudo-queen bioassay. Much less ODA was found on the thorax and abdomen of queens caged for five days with workers (Figure 2). The biologically attractive levels of retinue pheromone present on the legs of queens (ca. 10^{-3} Qeq) is of considerable interest in light of the attractiveness of queen "foot-print substance" (Juska, 1978).

CONCLUSIONS

Newly eclosed virgin queens have low or negligible levels of all five mandibular components, but by 6 days of age they contain levels of the decenoic acids that are about half those found in laying queens. HOB appears in virgin queens within 1 week after eclosion, with HVA levels appearing only after queens have mated. The enantiomeric composition of 9HDA strongly favored the *R* enantiomer, especially in mated queens. The amounts of the mandibular pheromone components were unaffected by confinement, either in banks or mailing cages. Storage of queens on Dry Ice caused no change in amounts of pheromone components, offering an alternative method of shipping queen mandibular glands for analysis.

In addition to recognition of queen presence, this blend (or selected combinations of components) is involved in worker orientation during swarming and inhibition of queen rearing (Winston et al., 1989) and may be involved in a myriad of other queen-controlled activities that maintain social cohesiveness in the colony. All of these functions must be reexamined in light of this chemical knowledge. With the identification of queen-produced semiochemicals, a

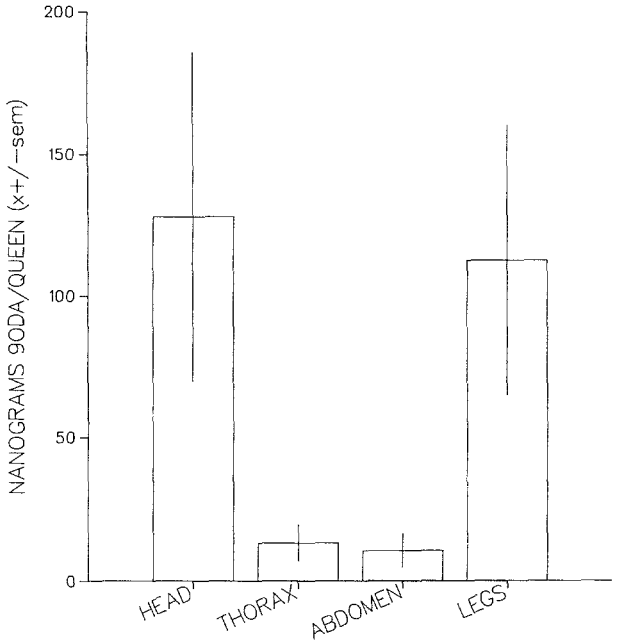

FIG. 2. Mean amounts of ODA found on the body parts of mated queens confined in a mailing cage with five workers for several days (N = 37).

fuller understanding of honeybee society is possible, with considerable potential economic benefits to be derived from an improved ability to manage and control this most complex of social insects.

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ISOLATION OF PHEROMONE SYNERGISTS OF BARK BEETLE, *Pityogenes chalcographus*, FROM COMPLEX INSECT-PLANT ODORS BY FRACTIONATION AND SUBTRACTIVE-COMBINATION BIOASSAY

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Abstract—Capillary gas chromatography with columns of different polarity and two-dimensional fractionation of effluents were used with novel subtractive-combination bioassays to rigorously isolate host- and insect-produced pheromone synergists of the bark beetle *Pityogenes chalcographus* (Coleoptera: Scolytidae). Methyl (*E,Z*)-2,4-decadienoate (*E,Z*-MD) and the previously identified chalcogran were found to be synergistically attractive to both sexes. *E,Z*-MD was produced sex-specifically in males, and only when they had fed on host-plant tissue. A Norway spruce monoterpene fraction (including α-pinene, β-pinene, and camphene) increased the attractive response to the pheromone components. Dose-response curves for *E,Z*-MD and chalcogran in the laboratory bioassay indicated the two components are highly synergistic. The isolation methods are important for further progress in identifying certain semiochemical synergists found in trace amounts in complex chemical mixtures, such as when insects must feed in host plants in order to produce pheromone.

Key Words—Pheromone, bark beetle, Coleoptera, Scolytidae, *Pityogenes chalcographus*, methyl (*E,Z*)-2,4-decadienoate, chalcogran, *Picea abies*, synergist, subtractive-combination bioassay, two-dimensional fractionation.

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INTRODUCTION

In Europe, *Pityogenes chalcographus* (Kupferstecher) is a serious pest of Norway spruce, *Picea abies* (L.) Karst., and is especially damaging to young trees. These tiny bark beetles (2 mm long) aggregate on certain host trees in response to a male-produced pheromone (Francke et al., 1977). Francke et al. (1977) isolated a unique spiroketal, chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane), from *P. chalcographus*, which, when released in the forest at 15 mg/hr, attracted conspecifics. They used a differential diagnosis method (Vité and Renwick, 1970) whereby the gas chromatographic elution patterns and mass spectra of chemicals are compared between the sexes for unique differences. About 100,000 beetles of both sexes were treated with juvenile hormone analog (ethyl-3,7,11-trimethyl-2-dodecenoate), but only the male was induced to produce chalcogran. However, since the natural release rates of chalcogran were not known and combinatorial bioassays of fractions in order to detect synergists were not performed, Francke et al. (1977) were not certain whether "a second component may be necessary for maximum response." Chalcogran also was not tested alone, but only with spruce bark (which is unattractive alone), so it was not known whether host volatiles play a role in the orientation response. Thus, we wanted to determine whether host-plant and/or other pheromone components participate with chalcogran in eliciting the attraction response of the beetle.

The problem with isolation of semiochemicals is that solvent extracts of insects or their volatile effluents may contain hundreds of compounds along with the few bioactive components of interest (Millar et al., 1985; Burger et al., 1985; Jackson et al., 1984; Birgersson et al., 1984; Byers et al., 1985) (Figure 1 below). In addition, many insects release trace amounts of pheromones and other semiochemicals only when feeding on host plants, which makes these isolations more difficult because of the complexity of the odors. Thus, in many isolation studies workers have chromatographically fractionated extracts of complex chemical mixtures into successively smaller portions until an active fraction in bioassay contains just a few compounds (Doss and Shanks, 1984; Jackson et al., 1984; Silk et al., 1985; Heath et al., 1986; Burger et al., 1985; Chuman et al., 1985; B.H. Smith et al., 1985; Löfstedt and Van Der Pers, 1985; Teal et al., 1985). These chemicals are usually identified by spectrometric methods, and then the corresponding synthetic compounds are bioassayed to confirm their behavioral role. However, these methods are inadequate for isolating synergistic components, where several in different fractions may be required together for biological activity, the usual case for the multicomponent insect pheromones (Silverstein and Young, 1976; Silverstein 1981).

With this synergistic problem in mind, the additive-combination bioassay of fractions was developed in the 1960s for the isolation of pheromone syner-

gists. Unfortunately, relatively few studies have used this methodology (Silverstein et al., 1966, 1967, 1968; Pearce et al., 1975; Millar et al., 1985) because it is laborious to test all combinations of many fractions for any number of possible synergists. L.E. Browne, while at the University of California, Berkeley, proposed a subtractive method for isolation of synergists (personal communication c. 1976). We have developed this novel idea into a subtractive-combination bioassay of gas chromatographic (GC) fractions, which is as rigorous as the additive method but requires far fewer tests. Our objective was to use this methodology to isolate from a complex, but natural, mixture of plant and insect volatiles all the pheromone synergists that are attractive to the six-spined spruce bark beetle *Pityogenes chalcographus*. If one or more additional pheromone components were discovered, we wanted to elucidate their synergistic properties with chalcogran.

METHODS AND MATERIALS

P. chalcographus beetles were reared in a laboratory culture originating from Lardal, Norway, on freshly cut Norway spruce logs at 27°C. At emergence, adults were collected and maintained at 4°C for several days until used in experiments (Anderbrant et al., 1990).

To determine whether host and/or other pheromone components may participate with chalcogran in eliciting the attraction response, we collected odors from air passed over appropriate host logs or beetle-infested host logs inside glass containers (Byers et al., 1985). Odor collections from logs infested with 40 males were made on 11 occasions as well as twice from uninfested host logs (from December 1982 to May 1985) to obtain enough material for bioassays and identification. Volatiles were collected from headspace air by passage through Porapak Q traps (300 mg, 80–100 mesh) at rates of 100–250 ml/min for two to five days, beginning one day after releasing males onto the logs. The absorbent was subsequently extracted with 2 ml diethyl ether to remove the trapped volatiles.

Each extract from a collection period was concentrated by allowing the diethyl ether to evaporate at room temperature from a tapered glass vial (W. Francke, University of Hamburg, personal communication) before further analysis or bioassay. The attractiveness of extracts and of various ratios of pheromone components were determined in a laboratory bioassay that tested the proportion of walking females in an olfactometer for their upwind responses to odor sources (Byers et al., 1985; Lanne et al., 1987). Beetles that did not respond the first time were given a second chance to reach the source. The light intensity in the bioassay arena was 180 lumens/m^2.

The subtractive-combination method of bioassay compares activity of the

whole blend of recombined fractions to the activities of all such blends, each with a different fraction missing or subtracted. If synergists occur, then one or more blends with a fraction removed will be found to have significantly less bioassay activity than does the whole blend. Thus, each of the removed fractions from these less active blends must contain at least one synergist. Further fractionation and subtractive-combination bioassays are then performed only with these "active" fractions (the fractions that were removed causing the loss in activity).

The concentrated extracts were analyzed on a Hewlett-Packard gas chromatograph (GC) model 5830 using a polar column (No. 1, Table 1) in order to formulate a sequence plan for fractionation by preparative GC. Fractionation was performed on a Carlo Erba model 4160 GC outfitted with a revolving microfraction collector (Wassgren and Bergström, 1984). The sequence GC fractionation of the whole extract is shown in Figure 1. A fused silica capillary

TABLE 1. GAS CHROMATOGRAPHIC COLUMNS[a] USED IN ANALYTICAL AND PREPARATIVE GC AND IN COUPLED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Analytical GC: Instrument: Hewlett-Packard, model 5830.
Polar GC column 1: Fused silica; length = 46 m, ID = 0.35 mm; OV-351, df = 0.59 μm.
Temperature program: 50°C for 5 min, 5°C/min to 200°C, then isothermal for 20 min.
Mobile phase: N_2 at 20 cm/sec.

Preparative GC: Instrument: Carlo Erba, model 4160.
Polar GC column 2: Fused silica; length = 24 m, ID = 0.35 mm; OV-351, df = 0.5 μm.
Temperature program: 50°C for 2 min, 5°C/min to 220°C, then isothermal for 20 min.
Mobile phase: N_2 at 30 cm/sec.
Nonpolar GC column 3: Fused silica; length = 26 m, ID = 0.32 mm; SE-33 cross-linked, df = 1.0 μm.
Temperature program: 80°C for 2 min, 5°C/min to 220°C, then isothermal for 20 min.
Mobile phase: N_2 at 30 cm/sec.

Analytical GC-MS: Instrument: Finnigan, model 4021.
Polar GC column 4: Fused silica; length = 23 m, ID = 0.20 mm; OV-351, df = 0.5 μm.
Temperature program: 50°C for 4 min, 8°C/min to 200°C, then isothermal for 10 min.
Mobile phase: He at 20 cm/sec.
Nonpolar GC column 5: Fused silica; length = 12.5 m, ID = 0.20 mm; SE-54 cross-linked.
Temperature program: 80°C for 3 min, 8°C/min to 220°C, then isothermal for 10 min.
Mobile phase: He at 20 cm/sec.

Quantitative GC-MS: Instrument: Finnigan, model 4021.
Polar GC column 6: Fused silica; length = 25 m, ID = 0.15 mm; Superox FA, df = 0.3 μm.
Temperature program: 50°C for 4 min, 8°C/min to 200°C, then isothermal for 10 min.
Mobile phase: He at 20 cm/sec.

[a]GC column 5, Hewlett-Packard, other GC columns prepared at Department of Chemical Ecology, Göteborg University.

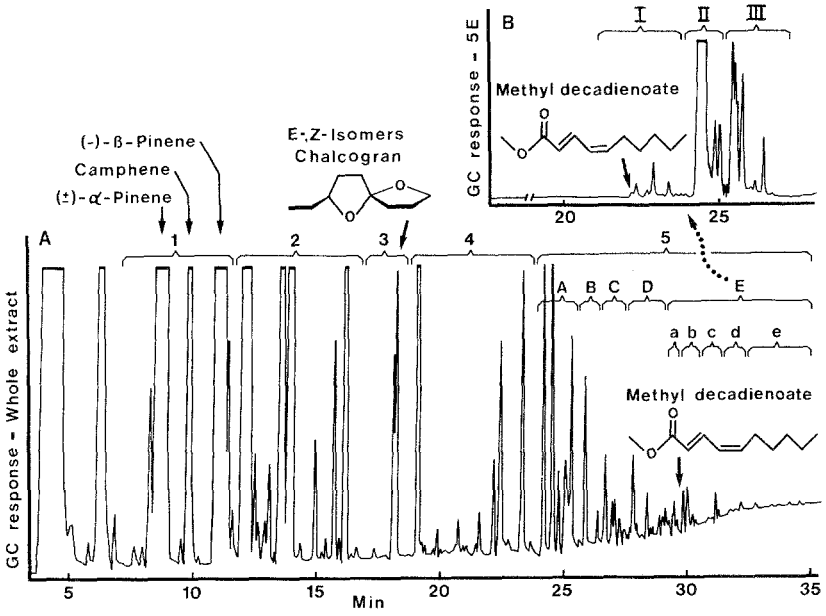

FIG. 1. Gas chromatograms (FID) of volatiles from a 40-male-infested Norway spruce log (25 × 8 cm diam.). Numbers and letters above peaks indicate the effluent collection periods for the respective fractions. Methyl (*E*,*Z*)-2,4-decadienoate cannot be discriminated visually in chromatogram A on a polar GC phase (column 1, Table 1) but was located by EICP-GC-MS and can be seen in chromatogram B as a small side peak (nonpolar GC phase, column 3).

column (No. 2, Table 1) coated with a polar stationary phase was used to obtain fractions 1–5, 5A–5E, and $5E_a$–$5E_e$ (Figure 1A). A second nonpolar GC column (No. 3, Table 1) was used to chromatograph fraction 5E, which contained the synergistic activity with chalcogran (Figure 1B). The identities of the indicated semiochemicals (Figure 1) were confirmed by matching their retention time and mass spectra with authentic standards using GC-mass spectrometry (GC-MS, columns 4–5, Table 1). Further details concerning the GC fractionation (Figure 1) and behavioral bioassays (Tables 2 and 3) used during the isolation of the synergists will be given with the results.

RESULTS AND DISCUSSION

The attractive activity of an extract of odor collection from a log infested with 40 males was compared to a similar extract of an uninfested log and to synthetic chalcogran in the bioassay. Volatiles from the infested and uninfested

logs were released in the olfactometer at the natural collection rates of 40 males/log/min and 1 log/min, respectively. The synthetic chalcogran (from W. Francke, University of Hamburg, West Germany), consisting of a racemic mixture of 46% (*E*)-(2*S*,5*R*- and 2*R*,5*S*-) and 54% (*Z*)-chalcogran (2*S*,5*S*- and 2*R*,5*R*-) stereoisomers, was released at 2 ng/min. The uninfested log extract or synthetic chalcogran alone were not appreciably attractive (both $< 10\%$ female response, $N = 30$–60 each) compared to the extract of volatiles from the male-infested log (73.3% response, $N = 30$, significantly different $P < 0.01$, χ^2). Natural chalcogran amounts were quantified in the extract and estimated to be released from the infested log in amounts up to 10 ng/min in the bioassay or by feeding males at up to 360 ng/beetle/day. A 0.1 rate of release (1 ng/min) from the infested logs of natural chalcogran (two stereoisomers, of which 0.46 ng was the active 2*S*,5*R*-isomer; Byers et al., 1989) still elicited a high female response (56.7%, $N = 30$) compared to the low response ($< 10\%$) to 2 ng synthetic chalcogran/min (four stereoisomers, of which 0.46 ng was the active 2*S*,5*R*-isomer. These results indicated that one or more synergists, in addition to chalcogran, elicit the attraction of the beetle. It was also possible that the 2*R*,5*S*- and 2*R*,5*R*-isomers of chalcogran were blocking response since the beetle does not produce these isomers (Schurig and Weber, 1984), and it has been shown that unnatural enantiomers can inhibit response in some species (Borden et al., 1976). However, in subsequent experiments (Byers et al., 1989), we have not found evidence of either synergistic or inhibitory interactions among the stereoisomers of chalcogran.

Using the subtractive-combination method of fractionation and bioassay (Figure 1A), we confirmed that natural chalcogran is an essential pheromone component of *P. chalcographus*. The combination of all fractions (1–5) released at 8 male/0.2 log/min attracted 65% ($N = 40$ females) but when fraction 3, containing chalcogran, was subtracted from the blend released at the same rate, the response dropped to 0% (Table 2). However, fraction 3 alone was not appreciably attractive (13.3%, $N = 30$). This is in agreement with the relatively low response to synthetic chalcogran reported above. Furthermore, the 65% response to the whole blend was reduced to 12.5% (Table 2) when fraction 5 was subtracted, indicating that this fraction contained one or more additional synergists. Only one other major fraction (No. 1) contributed to the attraction of the whole blend, as indicated by a slight decrease in response when subtracted (37.5% compared to 65% response, Table 2). This fraction contained the host-tree monoterpenes, α-pinene, camphene, and β-pinene (Figure 1A). These monoterpenes were shown later to play a role in host recognition by the beetle (Byers et al., 1988).

Thus, the subtractive bioassay required $1 + 5 = 6$ tests to isolate the synergistic activity in the major fractions (1–5). This is compared to the additive-combination method which would have required $1 + C_1^5 + C_2^5 + C_3^5 =$

TABLE 2. ATTRACTION OF FEMALE *Pityogenes chalcographus* IN THE LABORATORY BIOASSAY TO MIXTURES OF GAS CHROMATOGRAPHIC FRACTIONS (POLAR GC COLUMN 2, TABLE 1) OF AIRBORNE VOLATILE EXTRACTS FROM MALES BORING IN NORWAY SPRUCE LOGS

Stimulus[a]	Females (%) responding[b] (N = 30–40)	95% CI[c]
Fractions tested March 15, 1983 (cf. Figure 1A)		
1 + 2 + 3 + 4 + 5 = total blend	65.0a	49.5–77.9
Total blend − 1	37.5b	24.2–53.0
Total blend − 2	60.0a	44.6–73.7
Total blend − 3	0.0d	0.0–12.8
Total blend − 4	63.3a	45.5–78.1
Total blend − 5	12.5c	5.5–26.1
3 (natural chalcogran)	13.3c	5.3–29.7
Fractions tested April 23, 1983 (cf. Figure 1A)		
1 + CH[d] + 5A + 5B + 5C + 5D + 5E = blend		
Blend − 5A	63.3a	45.5–78.1
Blend − 5B	60.0a	42.3–75.4
Blend − 5C	50.0a	33.2–66.8
Blend − 5D	46.7a	30.2–63.9
Blend − 5E	10.0b	3.5–25.6
CH[d] + 5E	46.7a	30.2–63.9
Fractions tested May 10, 1983 (cf. Figure 1A)		
CH[d] + $5E_a$ + $5E_b$ + $5E_c$ + $5E_d$ + $5E_e$ = blend	50.0a	33.2–66.9
Blend − $5E_a$	50.0a	33.2–66.9
Blend − $5E_b$	46.7a	30.2–63.9
Blend − $5E_c$	46.7a	30.2–63.9
Blend − $5E_d$	50.0a	33.2–66.9
Blend − $5E_e$	40.0a	24.6–57.7
Fractions tested May 22, 1983 (cf. Figure 1A)		
CH[d] + $5E_a$	56.7a	39.2–72.6
CH[d] + $5E_b$	70.0a	52.1–83.3
CH[d] + $5E_c$	3.3b	0.6–16.7
CH[d] + $5E_d$	6.7b	1.8–21.3
CH[d] + $5E_e$	10.0b	3.5–25.6

[a] Release rate was 8 males/0.2 bolt/min.
[b] Values followed by the same letter within a test period were not significantly different ($\alpha = 0.05$, χ^2).
[c] Confidence interval for proportions.
[d] Synthetic chalcogran (46% E:54% Z) released at 2 ng/min.

26 tests to obtain the same information, assuming only three synergistically active fractions (1, 3, and 5 as shown here). However, if there had been 10 initial fractions and three synergists, then the subtractive method would have required 11 tests while the additive method would need 171 tests for the same level of isolation efficiency. However, even more tests than 171 would be needed if there were more synergists, in order to be assured of locating all the fractions with synergists.

In our initial fractionation, fraction 5 was clearly of importance in synergizing the activity of fraction 3 containing chalcogran. Thus, fraction 5 was subfractionated to further isolate the synergistic activity (when combined with chalcogran). As shown in Table 2, fraction 5 released at a rate equivalent to 8 male/0.2 log/min was little affected by the subtraction of fractions 5A–5D from the tested blends (47–63%), but the blend without 5E was of low attractiveness (10%). However, when fraction 5E was subfractionated, subtractive-combination bioassays could not determine which of the five fractions ($5E_a$–$5E_e$) contained essential components since all such blends with one of these fractions subtracted had a similar activity (40–50%, each $N = 30$). This implied that either there were at least two synergists (in addition to chalcogran) in different subfractions that were substitutive or, more likely, that one synergist had been split during fractionation between two consecutive fractions. The second hypothesis was supported when each of the five fractions ($5E_a$–$5E_e$) was tested alone with synthetic chalcogran. In these bioassays, both fractions $5E_a$, attracting 57% of the females, and $5E_b$, attracting 70% of the females, had significant activity compared to the other three fractions, $5E_c$–$5E_e$, each attracting $< 10\%$ of the females (Table 2). It was still possible that mutually substitutive synergists were present in these two fractions, although we know of no cases like this in species with three (or more?) synergistic pheromone components (Byers, 1989).

The GC elution of compounds of fractions $5E_a$ and $5E_b$ showed that several host sesquiterpenes, including γ-cadinene, δ-cadinene and α-curcumene, and myrtenol were present (GC-MS No. 6, Table 1). These compounds could have accounted for the loss of bioassay activity when the fractions were removed from the blend. Thus, volatiles were collected from uninfested host logs, and the extracts were fractionated, first on a polar column 2 to obtain 5E, and then on nonpolar column 3 (Table 1) to obtain two fractions. Each of these fractions were assayed at rates of 1.6 log/min with 4 ng/min of synthetic chalcogran. One host fraction, including δ-cadinene and myrtenol, elicited 13% response ($N = 60$), while the other fraction, with α-curcumene, elicited 6.7% response ($N = 60$), and the two fractions combined with chalcogran still only elicited 13.3% ($N = 30$) compared to a 50% response to the male infested log (fraction 5E with chalcogran) released at 8 male/0.2 log/min (significantly different, $P < 0.01$, χ^2). Since we could not discriminate differences in the GC elution

patterns of volatiles between fraction 5E from the host alone and fraction 5E from male-infested host logs, but the beetles could detect differences between responses to these fractions, we concluded that the beetle-produced synergist(s) needed to be further separated from the host constituents.

In order to separate the constituents of fraction 5E into a different GC elution pattern that might isolate the beetle-produced synergist(s), we utilized two-dimensional fractionation. We chromatographed fraction 5E (activity in $5E_{a+b}$) on nonpolar column 3 (Table 1, Figure 1B) and collected the effluent into three fractions (I–III). Our fractionation method is similar to the concept of two-dimensional GC described by Deans (1981), where the specific portion is reinitialized on the other column, i.e., all compounds begin at the same starting time. The synergistic activity of fraction 5E was found only in fraction 5E:I of three fractions (Figure 1B, Table 3). This fraction eluted prior to most of the sesquiterpene hydrocarbons. However, there were still several unidentified compounds in this fraction. By using a differential diagnosis technique, we compared every compound in fraction 5E:I of the nonpolar column to compounds eluting in the region between fractions $5E_a$ and $5E_b$ on the polar column using GC-MS extracted ion current profiles (EICP)(Chen, 1979; Garland and Powell, 1981). Only one compound (bp = 81; m/z = 111, 97% bp; M^+ = 182) was found both in the area of bioassay activity on the nonpolar (fraction 5E:I) and polar columns (split between fractions $5E_a$ and $5E_b$).

Once the active compound was located, more material was obtained from

TABLE 3. ATTRACTION OF FEMALE *Pityogenes chalcographus* IN LABORATORY BIOASSAY TO MIXTURES OF GAS CHROMATOGRAPHIC FRACTIONS (NONPOLAR GC COLUMN 3, TABLE 1) OF AIRBORNE VOLATILE EXTRACTS FROM MALES BORING IN NORWAY SPRUCE LOGS

Stimulus[a]	Females (%) responding[b] (N = 30)	95% CI[c]
Fractions tested April 3, 1984 (cf. Figure 1B)		
CH[d] + 5E:I + 5E:II + 5E:III = blend	60.0a	42.3–75.4
Blend − 5E:I	16.7b	0.7–33.6
Blend − 5E:II	53.3a	36.1–69.8
Blend − 5E:III	53.3a	36.1–69.8
CH[d] + 5E:I	40.0a	24.6–57.7
CH[d] + 5E:II	13.3b	0.5–29.7
CH[d] + 5E:III	13.3b	0.5–29.7

[a] Release rate was 8 males/0.2 bolt/min.
[b] Values followed by the same letter were not significantly different (α = 0.05, χ^2).
[c] Confidence interval for proportions.
[d] Synthetic chalcogran (46% *E*:54% *Z*) released at 2 ng/min.

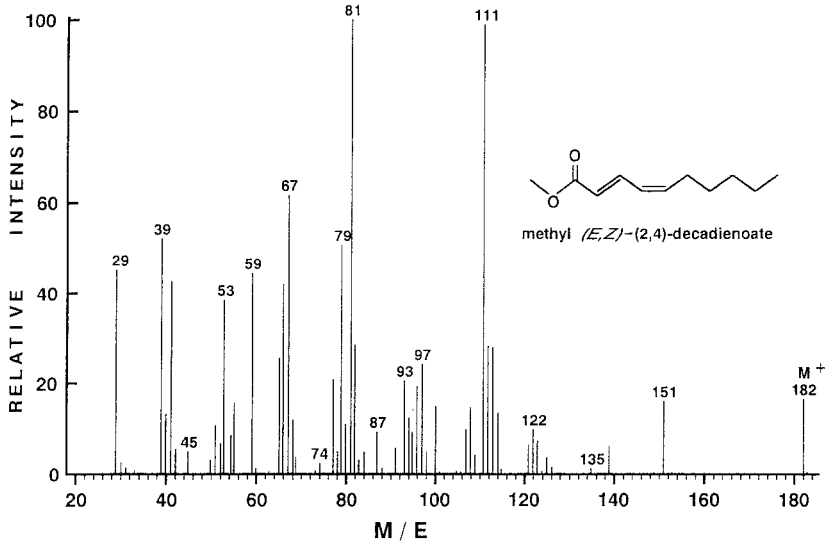

FIG. 2. Mass spectrum of methyl (*E,Z*)-2,4-decadienoate obtained from hindguts of 80 male *Pityogenes chalcographus* that had fed in a Norway spruce log for 42 hr (March 20, 1985). The molecular weight is indicated by M^+.

volatile collections and purified by preparative GC (columns 2 and 3, Table 1) to determine its mass spectra (Figure 2) and hydrogenation products. These spectra indicated the compound was methyl 2,4-decadienoate. Authentic standards of the four possible isomers [from reesterified ethyl (*E,Z*)-2,4-decadienoate, Oril produits chemiques, Neuilly-sur-Seine, France, or synthesized by R. Unelius, Department of Organic Chemistry, Royal University of Technology, Stockholm] were compared by GC-MS using retention times, coinjection, and MS data to prove that the isolated compound was methyl (*E,Z*)-2,4-decadienoate (*E,Z*-MD). *E,Z*-MD could not be found in extracts of host volatiles or in gut extracts of females feeding in the host for 48 hr. However, small amounts (about 10 ng/male) were located in feeding males but not in unfed males (Birgersson et al., 1990).

An unusual aspect of the two pheromone components of *P. chalcographus* is that they are acetogenic and not terpenic, as are most other bark beetle pheromones (Vanderwel and Oehlschlager, 1987; Byers, 1989). Esters such as *E,Z*-MD have, up to now, not been discovered in Scolytidae. *E,Z*-MD has not been identified before in insects and is a representative of a new class of semiochemical esters.

The synergistic properties of *E,Z*-MD and chalcogran mixtures were evaluated in the laboratory bioassay by varying each component over five orders of

magnitude in concentration while keeping the other component constant (Figure 3). *P. chalcographus* is exceptionally sensitive to both components, which is in agreement with the relatively low nanogram amounts released by males feeding in host logs. Recently we tested synthetic chalcogran and *E,Z*-MD in the forest (Byers et al., 1988), both together and alone, as well as together with the major host monoterpenes that were indicated to have activity in our initial fractionation (Figure 1A, fraction 1). We found that when chalcogran was released at 1 mg/day, it attracted few beetles (total of 39); when chalcogran was released with 18 μg *E,Z*-MD/day the catch increased by about 35-fold. The effect of the host monoterpenes, ($\pm$)-α-pinene, camphene, and ($-$)-β-pinene, when released in conjunction with the two pheromone components, was to increase the landing rate as well as induce both sexes to enter 2-mm-diameter holes in the artificial host (Byers et al., 1988).

Schurig and Weber (1984) found that two species of *Pityogenes* (*chalcographus* and *quadridens*) both contain the same stereoisomers of chalcogran. Apparently the beetles produce either (2*S*,5*R*)- or (2*S*,5*S*)-chalcogran or both

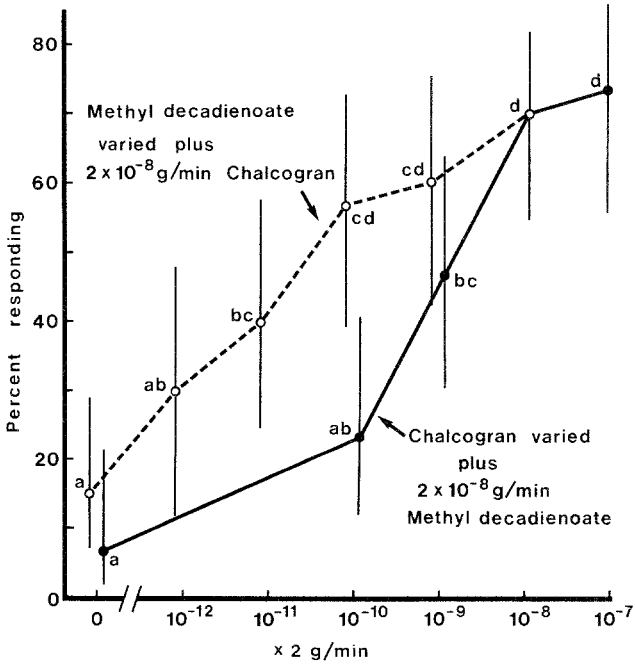

FIG. 3. Response of *Pityogenes chalcographus* to varying concentrations and ratios of its pheromone components, methyl (*E,Z*)-2,4-decadienoate and (46% *E* + 54% *Z*) chalcogran in the laboratory bioassay. The bars represent 95% confidence limits for proportions. Points with the same letter were not significantly different ($\alpha = 0.05$, χ^2).

but, in the acidic gut, they epimerize to the 46:54 ratio of 2*S*,5*R*:2*S*,5*S* isomers. *P. chalcographus* only contains these isomers of which the 2*S*,5*R*-isomer is the most active synergist with *E*,*Z*-MD, while 2*S*,5*S* is the least active (Byers et al., 1989). The other two possible isomers, 2*R*,5*S* and 2*R*,5*R* are not present in the beetle and are of intermediate activity. Pheromone discrimination between the *Pityogenes* species may reside in the *E*,*Z*-MD component or its isomers (*E*,*E*-, *Z*,*E*-, and *Z*,*Z*-). Recently, Baader (1989) investigated six species of *Pityogenes* (*chalcographus*, *quadridens*, *bidentatus*, *conjunctus*, *calcaratus*, and *carinulatus*) for volatiles released from attacked host material. Only the first two species produced chalcogran while only *P. chalcographus* released *E*,*Z*-MD.

Francke et al. (1977), in addition to (*E*)- and (*Z*)-chalcogran, found another male-specific compound, 1-hexanol, which had no apparent synergistic effect with chalcogran, although they stated that "it is also possible that the enantiomeric composition of the isomers (of chalcogran) is critical for maximum beetle response." In our samples, 1-hexanol was found in fraction 2 (Figure 1A), and this fraction did not exhibit synergistic activity since its subtraction from the whole extract did not significantly affect the attraction response.

Isolations of trace (nanogram) levels of synergistic pheromones have been accomplished in several species of moth (Roelofs and Cardé, 1977; Löfstedt et al., 1982, 1985), but these studies have often investigated relatively less complex odors from calling females alone. The number of compounds is often less than 20, but still there are numerous cases where additional pheromone components are discovered in well-researched species (Löfstedt and Van Der Pers, 1985; Teal et al., 1985; Silverstein et al., 1966, 1968; Roelofs and Cardé, 1977; Löfstedt et al., 1982, 1985; Booij and Voerman, 1985). More widespread adoption of our subtractive-combination method would facilitate the outcome of unequivocal studies with a minimum of work.

While multicomponent insect pheromones have become the paradigm (Silverstein and Young, 1976; Silverstein 1981), it seems that one-compound ideas are still ingrained in our "test each fraction" thinking about isolation of insect host stimulants (Kirk, 1985; Dicke et al., 1985; Stubbs et al., 1985; McKibben et al., 1985) and ovipositional stimulants (Maeshima et al., 1985; Kim et al., 1985; Hanula et al., 1985). In some of these cases there may be synergistic components, and without use of the subtractive-combination method there is the possibility of overlooking important components. Synergism between plant toxicants is known in plant–insect interactions (Berenbaum and Neal, 1985). Thus, the subtractive-combination method would also be a more efficient and systematic way of elucidating toxicant synergists. This also applies to isolating synergistic chemicals found in any biological or pharmacological system (cf. Norman, 1985).

A.B. Smith et al. (1985) have recently described a new approach for analysis of chemical signals using statistical methods of pattern recognition to detect differences between chromatograms. This is a variation of the differential diagnosis method (Vité and Renwick, 1970). However, these approaches presume that trace components can be discriminated by the particular chromatographic column. Also, these methods do not employ behavioral assays to determine which components are active either alone or, more importantly, in synergistic combinations. Thus, the subtractive-combination bioassay of chromatographic fractions is a more rigorous method that can be used to isolate all synergists, no matter when they elute and even if they are not visible with a particular chromatographic column used initially.

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EFFECTS OF MITE RESISTANCE MECHANISM OF GERANIUMS ON MORTALITY AND BEHAVIOR OF FOXGLOVE APHID (*Acyrthosiphon solani* KALTENBACH)

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Abstract—Geraniums (*Pelargonium xhortorum* Bailey) possess a pest-resistance mechanism, based on glandular trichomes and the exudate they produce, that has been shown to be effective against the two-spotted spider mite (*Tetranychus urticae* Koch). Using an intact plant bioassay, the effectiveness of the resistance mechanism was determined for another potential pest, the foxglove aphid (*Acyrthosiphon solani* Kaltenbach). Comparisons were made between plant lines previously analyzed for their degree of resistance to mites, as well as their glandular trichome density and trichome exudate production. Over 100 aphid adults were bioassayed on each of the five plant lines used in the experiment. In addition to adult aphid survival, the production and survival of nymphs was determined in this bioassay. The results indicate that plant lines that are resistant to the two-spotted spider mite are also resistant to the foxglove aphid, while lines susceptible to mites are susceptible to the aphids. To evaluate the physical impediment features of the trichome exudate, the behavior of foxglove aphid nymphs was compared on two geranium lines, one a resistant line with high trichome densities and large quantities of exudate and the second a susceptible line with few trichomes and reduced exudate. A third leaf surface type was produced by washing the exudate from resistant leaves using a mildly basic buffer solution prior to the bioassay. Aphid behavior was divided into five categories: feeding or probing, resting, wandering, struggling, and immobilized. On both susceptible leaves and resistant leaves from which the exudate had been removed by washing, the aphids settled quickly and were observed with inserted stylets during most of the observation intervals. In contrast, aphids on the unwashed resistant leaf

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surfaces often became ensnared in the sticky trichome exudate and had difficulty in settling to probe the leaf. Physical entrapment by glandular trichome exudate appears to be an important aspect of aphid resistance in geraniums.

Key Words—Glandular trichomes, geraniums, insect resistance, secondary compounds, *Pelargonium xhortorum*, foxglove aphid, *Acyrthosiphon solani*, Homoptera, Aphidae.

INTRODUCTION

In the geranium (*Pelargonium xhortorum* Bailey), tall glandular trichomes and the exudate they produce have been associated with resistance to the two-spotted spider mite and whiteflies (Craig et al., 1986; Gerhold et al., 1984; Stark, 1975). In a study by Winner (1975) with related plant lines, resistance was associated with two complementary dominant genes. Recently, it has been shown that resistant and susceptible plants differ in the number of tall glandular trichomes and the amount of exudate they produce (Walters, 1988; Walters et al., 1989). Both physical entrapment and toxicity have been suggested as modes of action for the exudate. In other plant species, glandular trichomes have been associated with both chemical and physical modes of insect resistance. Solanaceous species in particular are known to produce a variety of substances with toxic, repellent, and physical entrapment properties (Duffey, 1986; Gregory et al., 1986; Dimock and Kennedy, 1983).

In the course of research into the mechanism of resistance of geraniums to the two-spotted spider mite (*Tetranychus urticae* Koch), an infestation of the foxglove aphid (*Acyrthosiphon solani* Kaltenbach) was observed on some geranium plants in our greenhouse (Walters, 1988). The aphids were able to establish themselves and reproduce on selected geraniums. They preferred the young unexpanded leaf tissue at the apical shoot meristem, and their feeding produced innumerable lesions on the leaves. When the damaged leaves developed, they were malformed. Some plant lines appeared resistant to aphid attack, and no aphids were observed on the most mite-resistant lines even at times of heavy infestation among other geranium plants interspersed in the same greenhouse cubicle. In order to assess the effectiveness of the mite-resistance traits against other pests, bioassays were performed to see if plants resistant to the two-spotted spider mite were also resistant to the foxglove aphid. In addition, an experiment was conducted to examine the effects of the trichome exudate on the behavior of aphid nymphs confined to geranium leaf surfaces. By observing aphid behavior on a resistant line, a susceptible line, and a resistant line from which the exudate had been removed by washing with water (pH 9), we hoped to isolate the effects of the exudate from other potential influences on their behavior.

METHODS AND MATERIALS

Comparisons of Aphid Survival and Reproduction among Geranium Lines. This experiment was conducted in growth chambers (Environmental Growth Chambers; Chagrin Falls, Ohio) maintained at 23–26°C. Illumination was supplied by ten 40-W fluorescent bulbs and four 25-W incandescent bulbs, which produced a total flux of 280 μmol/sec/m^2 at the height of the plants. A photoperiod of 12 hr was alternated with 12 hr of darkness. Bioassays were performed on intact leaves of live plants. Five inbred geranium lines were assayed, three of which had previously been determined to be mite-resistant lines (71-17-7, 71-18-6, and 71-17-1) and two of which had been determined to be mite susceptible (71-15-4 and 71-10-1) (Gerhold et al., 1984; Stark, 1975; Winner, 1975). The aphids were obtained from an isogenic culture maintained on potato plants in an environmental growth chamber at 22°C with illumination provided by ten 40-W fluorescent and four 25-W incandescent bulbs. Two days prior to the bioassay, aphids were removed from the culture and isolated on an excised potato leaf with the petiole immersed in water to ensure that all insects were adults at the time of the bioassay. Two adult female aphids were caged on each leaf to be assayed using a fine mesh (158 μm pore size) polyester netting that allowed 50% of the incident light to reach the leaf. The netting was formed into envelopes, which were closed around the leaf petiole using ordinary cellophane tape. Two leaves were assayed on each plant for each trial. In order to minimize the effect of leaf age on the experiment, only leaves at the third node away from the apical meristem were used for the bioassay, except for a few instances when it was necessary to use a leaf at the fourth node. Leaves that were healthy in appearance and of similar size were used preferentially to minimize the effects of leaf vigor and size on the results of the experiment. Three vegetatively propagated plants of each line were assayed per trial, and the experiment was repeated nine times. The aphids were allowed to remain on the leaves for five days. At the end of the incubation period the leaves were removed from the plants, and the number of adult aphids still living was determined as well as the number of living aphid nymphs which they had produced. The counts of living nymphs thus reflected not only the number of nymphs produced, but also their ability to survive to the completion of the bioassay. In addition, the length and width of the leaves used for each aphid cage was recorded.

Mortality and living offspring data were analyzed using a two-level nested analysis of variance. The main factor consisted of the five inbred plant lines and the subordinate level was the individual plants (clones within lines) for each inbred line. An a priori comparison of the five plant lines was established before analysis of the data (Sokal and Rohlf, 1981). Three orthogonal comparisons were performed according to the following design: (1) resistant vs. susceptible; (2) line 10-1 vs. line 15-4; and (3) line 17-7 vs. line 17-1 vs. line 18-6.

Mortality data were recorded as the number of aphids living at the end of the five-day incubation. Aphids that could not be found either dead or alive were assigned a value of 0.5 for a neutral value. The number of nymphs was recorded directly. Data from the two individual leaves were combined and analyzed on a per plant basis so that the maximum number of living adults per observation was four. The data for both mortality and fecundity were transformed using the square root transformation ($Y^* = \sqrt{Y + 0.5}$) before the ANOVA was performed in order to reduce the dependence of the variance on the mean that is common with data of this sort (counts).

Behavior Bioassay. Aphid behavior was analyzed on three types of excised geranium leaf surfaces. Two separate geranium lines were tested: 17-7, a resistant line, and 10-1, a susceptible line (Gerhold et al., 1984; Stark, 1975; Winner, 1975; Walters et al., 1989). The aphids were also tested on resistant line 17-7 leaves from which most of the trichome exudate had been removed by washing for 10 min in 0.008 M carbonate–0.077 M bicarbonate, pH 9.0, buffer at 23°C. Thus by comparing aphid behavior on the washed versus unwashed resistant leaf surface, we could assess the exudate's effect on aphid behavior and also compare behavior on the susceptible leaves (line 10-1) with that on the resistant leaves (line 17-7) from which the exudate had been removed. Leaves were obtained from plants maintained in growth chambers under conditions described earlier to reduced environmental effects on leaf morphology and chemistry.

The leaves were placed abaxial side up in a Petri dish. Washed leaves were gently rinsed with tap water and carefully blotted dry with a paper towel to remove any trace of the buffer solution. Live first-instar foxglove aphids were placed in the center of freshly cut geranium leaves removed from the third node from the apical shoot meristem and observed under a dissecting microscope. The aphids were obtained from the culture already described. They were handled gently with a damp paint brush so as not to damage them or their mouthparts. After placing the insects on the leaf surface, a binocular dissecting microscope was used to observe them for individual periods of 1 min (observation periods) at 5-min intervals for 15 total intervals. Aphids that left the leaf surface were replaced and recorded. Thus the behavior was studied for 75 min after placing the insects on the leaves. Preliminary examination had shown that their behavior could be assigned to one of five categories as follows: (1) feeding/probing—mouthparts inserted in leaf, aphid stationary; (2) resting—mouthparts retracted, no attempted locomotion; (3) wandering—traversing the leaf surface largely unhindered; (4) struggling—attempting to traverse the leaf surface but spending more than 50% of the observation interval in nonproductive movements; and (5) immobile—unable to control movements.

During intervals when the aphids were not observed, they were removed from under the microscope illuminator (Fiberoptic Specialities Inc., model

LS81B) in order to reduce the effect of the bright light on their behavior. If a nymph left the leaf surface in favor of the Petri dish, it was replaced on the leaf and the event was recorded. Twenty replications of each of the three treatments were performed using a different aphid nymph and a fresh leaf for each trial for a total of 60 trials.

RESULTS AND DISCUSSION

The results of the intact plant bioassay indicate that geranium lines that are resistant to the two-spotted spider mite are also resistant to the foxglove aphid (Figure 1). Adult survival was lowest (< 1 aphid per plant) on line 17-7, which is also the most mite-resistant line. Aphids on the other two mite-resistant lines (18-6 and 17-1) also exhibited lower survival than aphids on the two susceptible lines. Analysis of variance of adult mortality data (Table 1) for the nine dates on which the experiment was repeated shows highly significant differences among the inbred lines. There were no significant differences among clones within lines for either the mortality or fecundity data. A priori comparisons for the mortality data indicate that almost 90% of the sum of squares among lines is due to the comparison between resistant and susceptible plant lines. There was no significant difference between the two susceptible lines analyzed, but there was among the three resistant lines, largely because of the high mortality shown on line 71-17-7. These data are supported by the morphologic and chem-

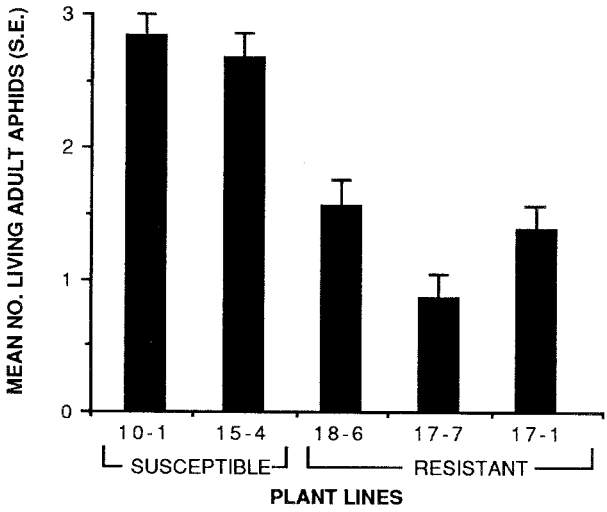

FIG. 1. Adult aphids remaining alive at completion of intact plant bioassay.

TABLE 1. ANALYSIS OF VARIANCE—APHID MORTALITY

Source of variation	df	SS	MS	Fs[a]
Among plant lines	4	10.857	2.714	24.2***
A priori comparisons				
Resist. vs. suscept.	1	9.522	9.522	84.9***
10-1 vs. 15-4	1	0.025	0.025	0.2NS
18-6 vs. 17-7 vs. 17-1	2	1.310	0.655	5.8*
Among plants within lines	10	1.948	0.195	1.7NS
Error	135	15.141	15.141	
Total	149	27.945		

[a]NS, nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ical comparisons performed on these same plant lines, which showed 17-7 possesses higher tall glandular trichome densities and produces more exudate than the other resistant lines (Walters et al., 1989).

As illustrated by Figure 2, the nymph data parallel that of the mortality data in every way. The number of surviving nymphs is lowest on mite-resistant line, 17-7, with less than three surviving nymphs per plant. In comparison, aphids caged on mite-susceptible lines produced five times as many nymphs that survived the duration of the bioassay. Living nymph numbers on resistant lines 17-1 and 18-6 were lower than the susceptible lines but higher than 17-7.

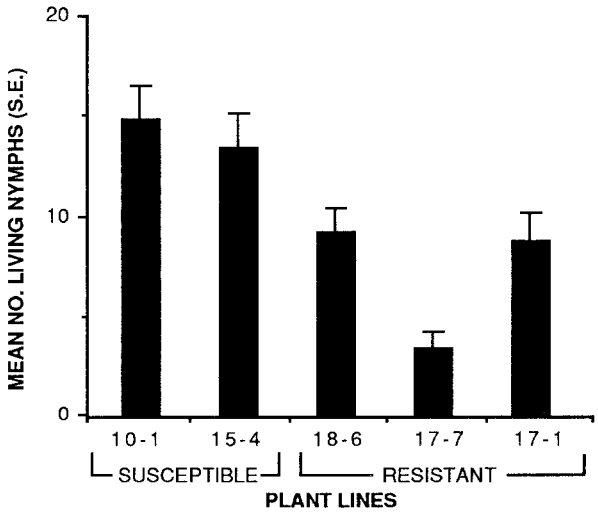

FIG. 2. Aphid nymphs alive at completion of intact plant bioassay.

TABLE 2. ANALYSIS OF VARIANCE—APHID FECUNDITY

Source of variation	df	SS	MS	Fs[a]
Among plant lines	4	71.312	17.828	11.0***
A priori comparisons				
Resist. vs. suscept.	1	43.820	43.820	26.9***
10-1 vs. 15-4	1	0.310	0.310	0.2NS
18-6 vs. 17-7 vs. 17-1	2	27.182	13.591	8.3**
Among plants within lines	10	16.475	1.648	1.0NS
Error	135	219.844	1.628	
Total	149	307.632		

[a]NS, nonsignificant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

In the analysis of variance of the fecundity data in Table 2, there are no significant differences shown among the clones within the geranium lines but the differences among the plant lines are highly significant. In other words, the responses of plants within a geranium line were consistent, while there were important differences among the lines themselves. As with the mortality data, the majority of the sum of squares among lines is the result of the comparison between resistant and susceptible plant lines with a lesser but still statistically significant proportion coming from the comparison among resistant lines. Again most of the latter contribution to the sum of squares comes from the low numbers of living nymphs on line 71-17-7, the other two lines being very similar to each other.

Results of the behavior bioassay are presented in Figure 3, which illustrates the distribution of the observation periods for all aphids on a given treatment. The most obvious differences concern the reduced feeding/probing exhibited by nymphs on 17-7 (44%) compared with that on 10-1 and the washed version of 17-7 (83% and 90%, respectively). Aphids on line 17-7 were struggling in 16% of the observation periods and immobilized 22% of the time, while the other two treatments produced no aphid behavior in these two categories.

Table 3 presents the data organized according to the behavior of individual aphids. Additional indications of the differences between the treatments are shown by the fact that, for 17-7, nine of the 20 nymphs observed became immobilized within a 75-min period, while no nymphs in either of the other two treatments became immobilized. Furthermore, while only five aphids placed on unwashed 17-7 were classified as feeding or probing the leaf at more than 80% of the observation periods, 16 of the 20 aphids on line 10-1 and 18 of the 20 aphids on washed 17-7 showed this high rate of feeding-related activity.

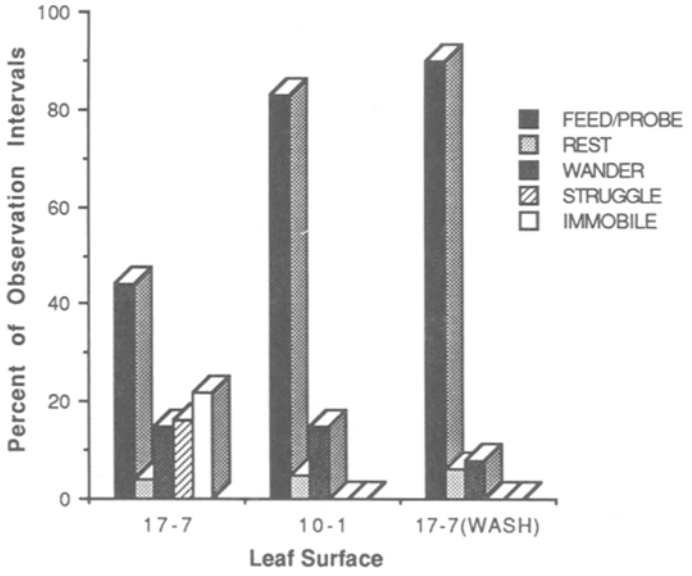

FIG. 3. Aphid behavior on excised leaves of resistant, susceptible, and treated resistant lines.

The behavior profiles presented in Figure 3 indicate that aphid behavior on line 10-1 and washed line 17-7 is very similar. This supports the hypothesis that the trichome exudate is an essential factor in providing resistance to the foxglove aphid. Plant line 17-7 possesses a high density of the tall glandular and tall spiny trichome types produced by *Pelargonium xhortorum* (Walters et al., 1989). Nymphs on the washed 17-7 leaves have a more difficult time traversing the leaf surface because of the higher density of trichomes, both glandular and nonglandular. Yet even in the presence of this "thicket" of epidermal appen-

TABLE 3. SUMMARY OF BEHAVIORAL OBSERVATIONS FOR FOXGLOVE APHIDS ON RESISTANT, SUSCEPTIBLE, AND TREATED RESISTANT GERANIUMS[a]

Treatment	No. of aphids immobilized	No. of aphids probing greater than 80% of observations	No. of aphids off leaf surface
17-7	9	5	13
10-1	0	16	4
17-7 (wash)	0	18	2

[a] Maximum possible value is 20.

dages, the nymphs are able to find an area to feed and they settle quite readily. It is only with the added impediment of the sticky exudate that the environment of the leaf surface becomes life-threateningly hostile to the insect.

The authors recognize that excised leaves may differ in important ways from intact tissue on living plants. Obviously, as far as aphid feeding is concerned, changes in phloem pressure and composition associated with leaf excision could have an important influence on the ability of aphids to feed. However, any physiological changes caused by excision would be expected to be very similar for the geranium lines and therefore would not explain the contrasting behavior patterns of aphids on these surfaces. Physiological changes do not explain why the removal of trichome exudate from resistant leaves causes aphids to exhibit behavior patterns nearly identical with those of aphids on the susceptible line.

Even on the resistant leaves, those insects that spent a minimal time wandering and quickly found a feeding site were able to survive, at least in the short term, by minimizing their exposure to the exudate. Those that performed an excessive amount of wandering found themselves in an ever-worsening situation, gradually accumulating a coating of viscous, sticky exudate that covered their legs and antennae. The appendages became stuck to one another and to glandular and nonglandular trichomes, requiring large amounts of energy to extricate the insect. This struggling caused even more contact with the exudate on the glandular trichomes, increasing the problems for the aphid nymph. Thus, in this short-term experiment it appears that the physical entrapment of small pests was more important than the toxic properties of the exudate. Physical-entrapment-based resistance is not a new phenomenon. It has been demonstrated in the pest resistance of wild tomato and wild potato species (Gentile et al., 1969; Gibson and Turner, 1977; Duffey, 1986; Gregory, 1986). The creosote bush and alfalfa also produce a glandular trichome exudate that functions to ensnare insect pests (Rhoades, 1977; Shade et al., 1975). Although the behavioral experiment was not repeated with adult aphids, observation indicates that they do not experience the same degree of entrapment as the young nymphs because of their larger size. Their longer legs allow them to avoid the exudate more easily, stepping over the impediments to which the smaller individuals fall prey. However, as shown here, even adult aphids experience increased mortality on the resistant leaf surface. Since aphids are essentially a permanent resident of the plant surface throughout their life cycle, high mortality among early instars increases the impact on aphid populations by preventing them from reaching reproductive maturity.

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COMPOUND INTERACTIONS EFFECTS OF PLANT ANTIOXIDANTS IN COMBINATION WITH CARBARYL ON PERFORMANCE OF *Trichoplusia ni* (CABBAGE LOOPER)

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Abstract—Plant chemicals naturally exist in complex mixtures, which can interact either additively, synergistically, or antagonistically. We investigated the potential interactions of three naturally occurring antioxidants—nordihydroguaiaretic acid (NDGA), safrole, and α-tocopherol—with the general insecticide carbarayl to affect the performance of cabbage looper larvae (*Trichoplusia ni*). The cabbage looper is known to produce a mixed-function oxidase enzyme system in response to the presence of carbaryl. We proposed that plant antioxidants would interfere with enzymatic oxidation, enhancing the susceptibility of this insect to carbaryl. Insects were fed artificial diets containing each antioxidant alone or in pairwise combinations with the insecticide carbaryl to test for their effects on the insect's nutritional measurement indices. The three antioxidants tested were not equally effective individually against insect survivorship and interacted differentially in combination with the insecticide. The nutritional indices were measured on insects fed diets containing the chemicals at nonlethal doses. Insects fed 0.001% wet wt NDGA diets grew 1.62 times less, and had gross and net conversion efficiencies reduced 3.20 and 3.63 times, respectively, compared to the control larvae. Carbaryl (0.002% wet wt) in combination with NDGA acts as an

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antagonist to the effects mentioned above, while safrole (1×10^{-4} wet wt) had an additive effect when combined with the insecticide, reducing 1.76 times larval relative growth rate and efficiency of conversion of ingested food in respect to the control. The larvae fed significantly more (1.2 times) on both insecticide and safrole diets than on the controls or their combined diets. Larvae fed α-tocopherol alone or in combination with carbaryl had similar growth and conversion efficiencies as controls. We conclude that the effects of different combinations of compounds cannot be predicted a priori and must be determined experimentally.

Key Words—Antioxidant, insecticide, cabbage looper, *Trichoplusia ni*, Lepidoptera, Noctuidae, antagonist, synergist, chemical heterogeneity.

INTRODUCTION

Plants produce a complex array of chemicals in their different tissues (Rosenthal and Janzen, 1979; Goodwin and Mercer, 1983). When ingested by an herbivorous organism, these mixtures of compounds can interact in affecting performance either additively, synergistically, or antagonistically (actually producing an effect less than the additive interaction) (Georghiou, 1983). Synergistic interactions (an enhancement over the additive interaction of two or more compounds) can take place between different plant allelochemicals as well as between these products and artificial insecticides. For example, pesticide synergists are compounds that serve to enhance the toxicity of a pesticide chemical when they are combined at nonlethal concentrations, generally by inhibiting the enzymes involved in insecticide detoxification (Metcalf, 1967).

Sesamin has been proposed as a natural synergist in *Crysanthemum cineraraefolium* flowers (Casida, 1970). The lignan myristicin and the linear furanocoumarin xanthotoxin from *Pastinaca sativa* had a synergistic effect on *Heliothis zea* (Berenbaum and Neal, 1985), as well as the methylenedioxyphenyl-containing amides from *Piper nigrum* on *Callosobruchus chinensis* (Miyakado et al., 1983). Natural methylenedioxyphenyl-containing compounds, including lignans, are widely distributed in plants (Vickery and Vickery, 1982), including *C. cineraraefolium*, the commercial source of pyrethrin insecticides (Ahmad et al., 1986).

These studies support the idea that plants may include synergistic mixtures in their chemical defenses (Berenbaum, 1985; Raffa and Priester, 1985). The implications of the chemical complexity of plants were first recognized by Janzen (1973), and later Jermy (1984) pointed out that there is no a priori reason to expect that single-chemical bioassays will be instructive when multicomponent defensive systems are operative in nature.

In this work, we examined the effect of nordihydroguaiaretic acid (NDGA), a common lignan catechol from the external resin of *Larrea tridentata*, on the

survivorship and nutrition of cabbage looper larvae (*T. ni*) fed artificial diets containing this chemical alone or in combination with the insecticide carbaryl. The antioxidant properties of this lignan led us to propose a possible synergism with a general toxin that is detoxified by the insect's oxidative microsomal enzymes. Both compounds were used at nonlethal doses because it is the chronic, rather than the acute, toxicities of most allelochemicals that, through their effects on insect growth, development, and reproduction, provide the more usual form of chemical defense in plants (Gunderson et al., 1986).

The cabbage looper was selected for the experiment because it is a polyphagous insect equipped with PSMO-detoxifying enzymes that can metabolize the insecticide carbaryl (Kuhr, 1971). The gut and fat body tissues of this organism contain cytochrome P-450 monooxygenase that is inhibited by methylenedioxyphenyl synergists (such as piperonyl butoxide, sesamin, and safrole) (Kuhr, 1971). We repeated the experiment using two other plant antioxidants to compare their effects: (1) the phenylpropene safrole(s), abundant in the essential oil of *Sassafras* spp. and a PSMO inhibitor in *T. ni* (Kuhr, 1971), and (2) α-tocopherol, which is the most biologically active of the four major tocopherols and an essential vitamin for insects, abundant in seed oils (wheat germ), and one of the most active "in vitro" chain-breaking antioxidants, (Burton and Ingold, 1981).

METHODS AND MATERIALS

Experimental Design: Freshly hatched larvae of *Trichoplusia ni* from a laboratory colony were placed in individual lidded cups (Solo Cup Co.) with approximately 4 g of wet diet (Bioserv. Inc. Cabbage Looper Diet Product No. F928Z), containing the following antioxidants alone or in combination with the insecticide.

Chemicals. As plant-occurring antioxidants, we selected the phenylpropene safrole, NDGA (a lignan antioxidant), and α-tocopherol (Sigma Chemical Co.). These chemicals were used alone or in pairwise combinations with the insecticide carbaryl (1-naphthylmethylcarbamate, extracted with acetone from commercial 5% Sevin, Blakleaf Products Co.), in artificial diets.

The concentrations of each compound used for the dose–response bioassay were 1.00%, 0.10%, 0.002%, 0.001% (plus 0.00001 and 0.00002% for safrole) wet weight. Each experiment consisted of 15 replicates and was run at 21–24°C on a 12 : 12 light-dark schedule. Water loss was constant for all experimental and control cups, which were kept at the same temperature and relative humidity regimes. Larvae were scored for: (1) daily survivorship and (2) wet weight of diet, frass, and larvae at the beginning and end of the experiment (14 days). The following feeding indices were calculated from larval wet weight

using the formulae of Waldbauer (1968):

$$\text{RGR (relative growth rate)} = \text{Ln [(final larval weight/initial larval weight)/time of experiment]}$$

$$\text{ECD (efficiency of conversion of digested food)} = \frac{\text{weight gained by larvae}}{\text{weight food eaten} - \text{weight frass}} \times 100$$

$$\text{ECI (efficiency of conversion of ingested food)} = \frac{\text{weight gained by larvae}}{\text{weight food eaten}} \times 100$$

$$\text{AD (approximate digestibility)} = \frac{\text{weight food ingested} - \text{weight frass}}{\text{weight food ingested}} \times 100$$

After a survivorship analysis of the dose–response experiment (Survivorship Program, SPSSX 2nd ed. 1986) a concentration of 0.001% wet wt of antioxidants (1×10^{-4} for safrole) was selected to be used in combination with carbaryl (0.002%) for the second set of feeding experiments. For the combined diets, we selected concentrations that gave survivorship values over 80% to avoid excessive larval mortality: carbaryl, NDGA, and α-tocopherol, 0.001%; safrole, 0.0001%.

RESULTS

The survivorship of larvae was significantly affected by all the treatments with concentrations in the diets of 0.01% wet wt or larger (Figure 1-4) with safrole exhibiting the greatest toxicity among the chemicals used. To achieve survivorship levels with safrole similar to those obtained with the lowest concentrations used with the other six treatments, a 1 : 100 dilution was required (Figure 3). The survivorship of larvae fed diets with the different pairwise combinations of chemicals did not differ from control larvae or those fed individual chemicals.

Feeding indices were calculated for larvae fed diets containing the selected concentrations of each antioxidant, the insecticide, and their combinations (Table 1). Carbaryl (C), safrole (S), tocopherol (T), and carbaryl plus NDGA (CN) -containing diets did not affect any of the calculated feeding indices. Insects fed on NDGA (N) diets grew 1.62 times less and had gross and net

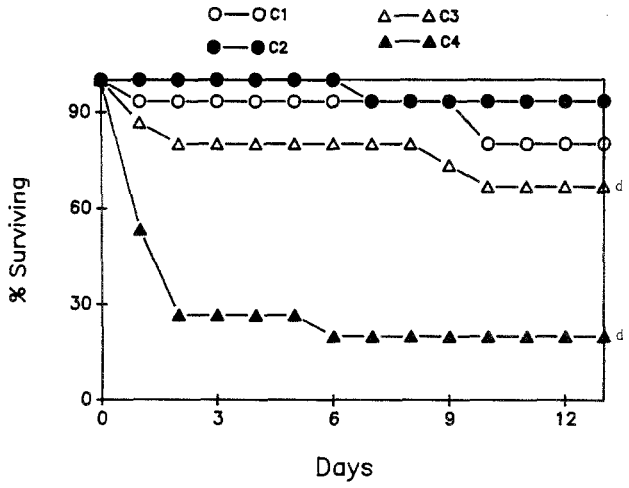

FIG. 1. Survivorship curves for *T. ni* larvae growing on carbaryl-containing diets. The concentrations of the chemical in the diets are: C_1, 0.001%; C_2, 0.002%; C_3, 0.01% and C_4, 0.10% wet wt. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; and d, $P < 0.001$: significance levels when the values are statistically different from the control. *$P < 0.05$: significance level when the values between concentrations are statistically different.

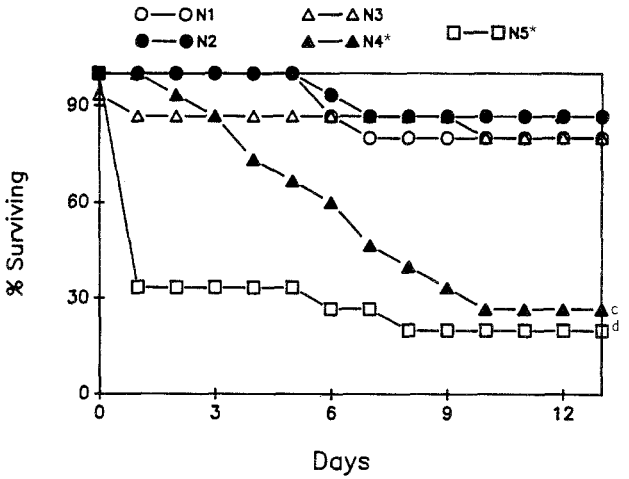

FIG. 2. Survivorship curves for *T. ni* larvae growing on NDGA containing diets. The concentrations of the chemical in the diets are: N_1, 0.001%; N_2, 0.002%; N_3, 0.01%; N_4, 0.1% and N_5, 1.0% wet wt, and the significance levels are as in Figure 1.

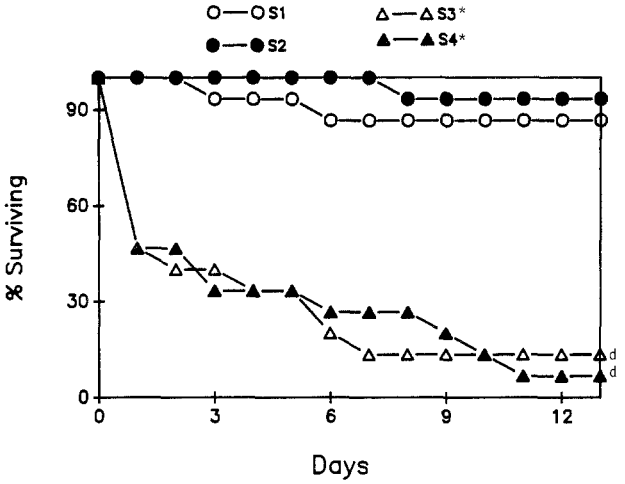

FIG. 3. Survivorship curves for *T. ni* larvae growing on safrole-containing diets. The concentrations of the chemical in the diets are S_1, 0.00001%; S_2, 0.00002%; S_3, 0.001% and S_4, 0.01% wet wt, and the significance levels are as in Figure 1.

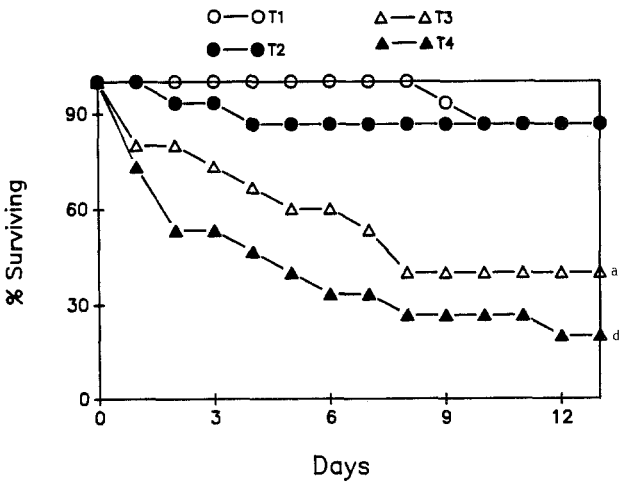

FIG. 4. Survivorship curves for *T. ni* larvae growing on α-tocopherol-containing diets. The concentrations of the chemical in the diets are: T_1, 0.001%; T_2, 0.01%; T_3, 0.1% and T_4, 1.0% wet wt, and the significance levels are as in Figure 1.

conversion efficiencies reduced 3.2 and 3.63 times, respectively, when compared with the control larvae. The carbaryl + safrole-fed larvae had both relative growth rate and gross conversion efficiencies reduced 1.76 times.

To determine if there were any interactive effects other than additive as a result of the combination of the insecticide and the antioxidants, we defined "interaction" as the effect of the combined diet being different from the sum of effects from each one of the components present in the combination. We tested this postulate by rejecting the null hypothesis of the pairwise comparison [(control) − combination (A + B)] and [(control − A) + (control − B)], at probability level of 95% for each one of the feeding indices calculated. The CN diet was the only combination that resulted in a significant nonadditive "interaction," for all the feeding indices (Table 1).

The N diets reduced the relative growth rate and approximate digestibility of the larvae 1.46 and 0.96 times, respectively, when compared with insecticide-fed larvae, but did not show any significant differences for either gross or net efficiencies. Larvae fed N diet also had relative growth rates, gross and net conversion efficiencies, and approximated digestibility reduced 0.70, 0.40, and 1.04 times, respectively, when compared to those fed on CN diets, while C and

TABLE 1. FEEDING INDICES OF *T. ni* LARVAE FED DIFFERENT DIETS[a]

	Control	C	N	S	T	CN	CS	CT
RGR								
$\bar{X}$	1.27	1.08	0.78d[b]	1.33	1.24	1.12[c]	0.72c	1.07
SD	0.06	0.08	0.09	0.09	0.06	0.09	0.15	0.09
ECI								
$\bar{X}$	6.41	3.66	2.00d	6.08	4.80	4.91[c]	2.91a	3.83
SD	3.43	2.20	1.73	4.83	1.93	3.74	3.45	2.61
ECD								
$\bar{X}$	7.68	4.06	2.11d	6.32	5.30	5.58[c]	3.18	4.20
SD	4.70	2.67	1.37	5.73	7.22	4.82	3.64	3.30
AD								
$\bar{X}$	87.5	92.5	96.3d	91.8	91.6	92.3[c]	94.4	94.6a
SD	7.7	4.3	3.1	4.5	3.3	5.7	7.6	5.4
Sample size	13	15	14	11	13	14	13	12

[a] Diets contain NDGA (N) and α-tocopherol (T) at 0.001%, carbaryl (C) at 0.002%, and safrole (S) at 0.00001% wet wt. Combination diets (CN, CS, and CT) were additive for the amounts in the single compound diets. RGR, relative growth rate; ECI, gross conversion efficiency; ECD, net conversion efficiency; and AD, approximate digestibility.

[b] a, $P < 0.05$; b, $P < 0.01$, c, $P < 0.005$; and d, $P < 0.001$ significance levels for values statistically different from the control.

[c] $P < 0.05$ significance level of the pairwise comparison between [(control) − combination (A + B)] and [(control − A) + (control − B)] for the feeding indices calculated.

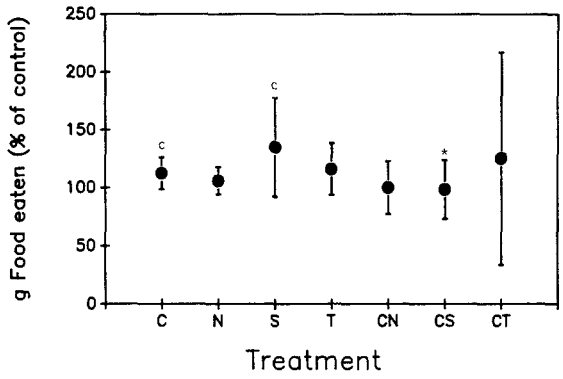

FIG. 5. Amount of food eaten by *T. ni* larvae growing on artificial diets. The concentrations of the chemicals used and the treatments are the same as in Table 1. c, $P < 0.005$: significance level when the values are statistically different from the control. *$P < 0.05$ significance level when the combined diet values are statistically different from those of each corresponding single-component diet.

CN diets did not show any significant differences between their effects on the feeding indices (Table 1). NDGA had a strong negative effect on all feeding indices (except for AD), while its combination with the insecticide gave values higher than those from each one of the components separately.

Grams of food eaten by the insects during the experiment were expressed as the percentage of food eaten of the control diets (Figure 5). The larvae consumed significantly more diet on both C and S diets than on the controls (Figure 5). These insects consumed an average of 1.2 times more food than the insects fed control and the CS diet. Insecticide-fed larvae also consumed more food than NDGA-fed larvae (1.05 times), and none of them larvae fed (C and N diets) consumed significantly more or less than those fed CN diets (Figure 5).

DISCUSSION

Plant antioxidants, such as safrole, can inhibit the detoxification enzymes of some herbivorous insects (Kuhr, 1971), thus acting as insecticide synergists. We proposed that NDGA, a strong plant antioxidant, could exhibit either an additive (total effect is equal to the sum of the parts) or synergistic (greater effect than the sum of the parts) effect in combination with a general toxin such as carbaryl, when fed to *T. ni* larvae.

NDGA, a lignan catechol, is the major component of the external resin that coats *Larrea tridentata* (Zygophyllaceae) leaves and stems (Rhoades, 1977b). The resin has antiherbivore properties, deterring leaf-chewing insects,

which prefer to eat low-resin-content leaves and stems (Rhoades, 1977a,b), with NDGA being an important resin component. Feeding trials have shown that *Ligurotettix coquiletti*, a grasshopper associated with *L. tridentata*, preferred foliage from bushes that contained low amounts of NDGA (Greenfield et al., 1987; Chapman et al., 1988). Additionally, NDGA has been shown to negatively effect survivorship of *T. ni* larvae fed artificial diets (Gonzalez-Coloma et al., 1988).

NDGA has also been described as a phototoxin (Downum and Rodriguez, 1986) and protects *Larrea* shrubs from ozone damage, presumably by forming a surface film of ozonized NDGA, as ozone fumigation significantly reduced the NDGA content of exposed *Larrea* resins (Gonzalez-Coloma et al., 1988). Rubin et al. (1980) proposed inhibition of the polysubstrate monooxygenase (PSMO) to explain the protection that antioxidants offer to plants against ozone injury. This is not the case for NDGA in *Larrea* resins since it is a superficial compound but, for the same reason, it could interfere with the PSMO system of the herbivores, acting as a synergist, thus enhancing the protective role that NDGA plays in *L. tridentata* shrubs.

The negative effect that all the chemicals used in this experiment had on *T. ni* larvae was not uniform, ranking as follows: safrole > carbaryl > NDGA = α-tocopherol. Safrole has been reported as being very toxic to insects (Yu, 1987), carbaryl and NDGA have been previously described as having negative effect on *T. ni* survivorship (Kuhr, 1971; Gonzalez-Coloma et al., 1988), and α-tocopherol may affect larval survivorship because of its lipophilic nature (Casida, 1970).

In contrast to the rank-order effectiveness of these compounds on insect survivorship, we found a different ranking of greatest to least effect at nonlethal doses on *T. ni* feeding indices, suggesting different mechanisms of action. Among the single-component diets, only NDGA had a significant effect on all the insect feeding indices tested when compared to the controls. NDGA diets decreased relative growth rate and conversion efficiencies of *T. ni* larvae. Larvae were not deterred by NDGA presence, as similar amounts of food were consumed in NDGA and control diets.

Rhoades (1977b) proposed that NDGA has protein- and starch-complexing as well as digestibility-reducing properties like those of tannins. These compounds limit larvae of *T. ni* via reduced digestibility, changing their assimilation efficiency (Wisdom et al., in press). The lowering of net conversion efficiency rather than the approximated digestibility of *T. ni* does not agree with this mechanism of action proposed for NDGA. Greenfield et al. (1989) have found the same effect on *L. coquilletti* that consumed *L. tridentata* foliage with a high NDGA content and suggested that this may derive from the intimate evolution of *L. coquilletti* with *L. tridentata* and adaptations for circumventing the tannin-like properties of NDGA (Bernays and Chamberlain, 1980) but not its toxicity.

Potentially toxic properties of this chemical, such as causing of midgut lesions in a manner similar to that of protein binding of tannins to gut tissues (Bernays et al., 1981; Steinly and Berenbaum, 1985), could act on metabolic processes since the replacement of damaged gut cells depends on the metabolic rate (Van Frankenhuyzen and Nystrom, 1987). In general, dietary and environmental changes may influence an organism's ECD (respiration losses) more than its AD on a particular foodstuff (Mattson, 1980).

When NDGA and carbaryl were combined, the effects observed gave a significant level of interaction for all the feeding indices tested. Contrary to our prediction, larvae of *T. ni* fed on this combination performed better than on N diets. This interaction can be considered an antagonism, where the activity of a mixture is less than that of the more active constituent (NDGA) (Metcalf, 1967).

There are many examples of enhanced resistance to insecticides by plant allelochemicals inducing the insect's detoxifying enzymes (Gould et al., 1982; Yu, 1987). This antagonistic effect of NDGA plus carbaryl did not involve metabolic cost, since we did not observe a significant reduction in the larval net conversion efficiency. Mechanisms other than enzyme induction may be involved in this type of effect; for example, the tobacco budworm, *Heliothis virescens*, is more effectively controlled by fenvalerate on cotton varieties with a high tannin content than on low-content varieties (Schuster et al., 1983).

In contrast, safrole, a well-known PSMO inhibitor and substrate (Casida, 1970; Kuhr, 1971; Yu, 1987), had an additive effect with carbaryl that did not result in the expected significant synergistic interaction, probably because under these experimental conditions the insects did not get the minimum concentrations of the chemicals needed to undergo metabolic changes. These CS diets were eaten significantly less than any of the corresponding single-component diets. This could account for the relative growth rate reduction observed in the larvae fed CS diets and their variation in ECI compared to the controls. An imposed reduction of food consumption (e.g., presence of a feeding suppressant) results in changes in AD, ECD, and RGR (see Scriber and Slansky, 1981).

These results emphasize the importance of interactive effects among secondary chemicals and the ecological relevance of the larvae's ability to compensate for variability in its food (see Scriber and Slansky, 1981). Lindroth and Peterson (1988) emphasize that, at ecologically relevant doses, the toxicity of phytochemicals is not an inherent trait, but minor structural changes within a compound may mask reactive sites, change solubility, or alter susceptibility to enzymatic degradation, all of which hold implications for herbivore performance and the dynamics of plant–insect interactions. For example, gossypol can interact antagonistically with phospholan against *Spodoptera littoralis* larvae when the two compounds are applied separately in water, yet they can interact synergistically if formulated in 0.01% NH_4OH (Meisner et al., 1977).

An unspecified compound has also been detected in *L. tridentata* resins from high-NDGA-content shrubs, which appears with very low frequency in low-NDGA-content ones (Gonzalez-Coloma and Greenfield, unpublished data). This group of plants shows no toxic effects on *L. coquiletti* (Greenfield et al., in press), the rest of the resin components being constant in each shrub type. The presence of this compound in the resin seems to enhance the effects that NDGA has on this specialist insect.

Finally, plants not only produce a particular compound but also specific quantities of each compound. Plant species vary both qualitatively (the type of compound produced) and quantitatively (the amount of each compound produced). Consequently, when measuring the interactive effects of compound mixtures, it will be important to determine the effect of the specific ratios of compounds as expressed in the plant. The types of interactive effects described here could potentially differ with the changing total concentrations and ratios of compounds found in plants.

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EFFECTS OF FERULIC ACID ON *Glomus fasciculatum* AND ASSOCIATED EFFECTS ON PHOSPHORUS UPTAKE AND GROWTH OF ASPARAGUS (*Asparagus officinalis* L.)[1]

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Abstract—The effect of ferulic acid, an allelochemical produced by asparagus, on hyphal elongation and colonization of asparagus by *G. fasciculatum* was studied. Spore germination in vitro was not affected, but hyphal elongation decreased significantly with increasing ferulic acid concentration. In the greenhouse, mycorrhizal colonization of roots and growth of mycorrhizal asparagus decreased significantly with increasing ferulic acid concentration, while growth of nonmycorrhizal plants was not affected by ferulic acid. Although plant tissue phosphorus levels were not affected by ferulic acid or mycorrhizal status, ferulic acid inhibition of hyphal elongation in vitro and fungal root colonization in vivo suggests that production of ferulic acid by asparagus reduces the symbiotic effectiveness of the fungus and subsequently reduces plant growth.

Key Words—Vesicular-arbuscular mycorrhizae, *Glomus fasciculatum*, allelochemical, asparagus, *Asparagus officinalis*, ferulic acid, fungus.

INTRODUCTION

Allelochemicals can suppress plant growth by interfering with the formation of mycorrhizae (Becker and Bennett, 1980; Chu-Chou, 1978; Del Moral et al., 1978), and by limiting the survival of effective fungal symbionts (Persidsky et al., 1965; Kovacic et al., 1984). Asparagus produces allelochemicals which are also autotoxic (Hartung, 1987; Yang, 1982; Young, 1984). Hartung (1987)

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isolated and characterized several allelochemicals from asparagus root tissue, including ferulic acid.

Ferulic acid can inhibit phosphorus uptake in plant roots (Glass, 1973; McClure et al., 1978). Exudation of ferulic acid by asparagus roots could affect colonization by vesicular–arbuscular mycorrhizal (VAM) fungi by negating the improved phosphorus nutrition commonly associated with VAM symbiosis. Vesicular–arbuscular mycorrhizal asparagus seedlings are larger than nonmycorrhizal plants (Chang, 1984; Hussey et al., 1984; Powell and Bagyaraj, 1983; Wacker, 1988). However, ferulic acid production in asparagus seedlings is low compared to older plants (>5 years; Hartung, 1987) and may not be at concentrations that would affect VAM colonization. This study was designed to test the hypothesis that ferulic acid is capable of inhibiting VAM fungal growth and colonization of asparagus roots, and to determine what effect, if any, this inhibition would have on phosphorus uptake and plant growth.

METHODS AND MATERIALS

Germination and Hyphal Elongation. A modified version of a root exudate medium that stimulates VAM hyphal elongation (Elias and Safir, 1987) was prepared. This medium contained (per 100 ml of glass-distilled water): 2 g Difco agar, 5 ml Hoagland's solution (10 × strength, without phosphorus), and 5 ml of rehydrated clover root exudate (taken from 1-week-old plants grown axenically in Hoagland's solution, then lyophilized and stored frozen until ready for use). The pH was adjusted to 6.8 and the medium autoclaved for 20 min at 121°C. Ferulic acid (final concentrations of 0, 50, 200, and 400 μg/g) was dissolved in 10 ml glass-distilled water (this required steaming for 1 hr). Ferulic acid solutions were Millipore-filtered (0.45 μm pore size) and added to the sterile root exudate medium and the pH was readjusted to 6.8. Ten milliliters of the ferulic acid–clover root exudate medium was added to each 60 × 15-mm Petri plate.

Glomus fasciculatum (Thaxt. sensu Gerd.) Gerd. and Trappe was grown in sorghum (*Sorghum vulgare*) pot cultures in the greenhouse for four months and stored at 4°C for four months prior to use. Chlamydospores were isolated from the soil surrounding the sorghum roots by wet sieving, followed by a modified centrifugation–flotation technique (Ohms, 1957). This modification involved the formation of a density gradient using 15, 30, 45, 50, and 60% Ficoll (Sigma Chemical Co., St. Louis, Missouri) to separate the chlamydospores from the soil particles. Chlamydospores were collected in a 38-μm cup sieve and washed several times with distilled water. Organic debris was further removed by hand from the final spore suspension using a Pasteur pipet under a dissecting microscope. Spores were surface-sterilized in 30 ml of a solution

containing 2% (w/v) chloramine-T, 0.02% (w/v) streptomycin sulfate, and a small amount of sodium laurel sulfate under vacuum for 30 min and washed with sterile distilled water (MacDonald, 1981). Chlamydospores were stored at 4°C in a solution containing 0.25% (w/v) streptomycin sulfate and 0.17% (w/v) gentamycin sulfate for use within five days.

Five spores were added per Petri plate at each concentration of ferulic acid using a sterile Pasteur pipet. There were 10 plates per ferulic acid concentration (50 spores per treatment). After the spores were added, the Petri plates were sealed with Parafilm and incubated at 30°C. Hyphal growth was measured every seven days for six weeks. Spores were considered germinated if the hyphal length equaled the diameter of the spore. Final growth data were log-transformed and analyzed by linear regression. This experiment was repeated once with similar results.

Growth of Asparagus With and Without VAM. Asparagus seeds (*Asparagus officinalis* L., var. UC 157) were surface-sterilized for 30 min in a 0.5% sodium hypochlorite solution and rinsed several times with sterile glass-distilled water. Seeds were added to a flask with 5 g benomyl (10 g Benlate 50WP, Dupont, Wilmington, Delaware) and 100 ml acetone and placed on a rotary shaker for 24 hr. Seeds were rinsed twice with 100 ml acetone, then rinsed with 100 ml sterile glass-distilled water six times (Damicone et al., 1981). Seeds, placed on sterile, moist filter paper, germinated within seven days. Germinated seeds were planted into 4-in.-diameter pots containing Baccto greenhouse mix (Michigan Peat Co., Houston, Texas). Asparagus plants were fertilized each week with 100 ml of NPK at 100 μg/g each. After four months, plants were transplanted into a steam-sterilized 2:1 (v/v) sand–soil mix in 5-in-diameter pots (one plant per pot). Half the plants received 1 g of *G. fasciculatum* inoculum (containing 50–60 spores/g soil, placed 1 cm below the roots at time of transplanting), and the remaining half was inoculated with 1 g autoclaved inoculum soil. Inoculum soil was wet-sieved through a 38-μm sieve and the wash collected in a beaker; inoculum wash contained microbes associated with VAM pot cultures that would have been eliminated in autoclaved inoculum soil. Both mycorrhizal and non-mycorrhizal treatments received 1 ml of inoculum wash. Ferulic acid solutions (0, 50, 100, 200, and 400 μg/g) were added to pots at a rate of 100 ml/week. There were five plants per treatment, arranged in a complete randomized block design. Plants were harvested at eight months of age.

Roots (0.5 g/plant) were removed at random and stained with a 0.1% solution of acid fuchsin in lactophenol (Phillips and Hayman, 1970). Forty root segments (1-cm lengths) per treatment were analyzed for VAM infection using the following rating system: 1 = no infection, 2 = 1–25% root segment infected, 3 = 25–50%, 4 = 50–75%, and 5 = over 75% infection. Dry weights were used to measure growth differences. Tissue phosphorus concentrations were obtained using Bartlett's technique (1959). The percent dry weight of tis-

sue phosphorus was calculated after obtaining the approximate concentration (micrograms per gram) in each sample by comparison to a standard curve. Infection ratings and growth data were analyzed by linear regression. Tissue P data were subjected to analysis of variance. This experiment was repeated once with similar results.

RESULTS

Germination and Hyphal Elongation. Spore germination continued throughout the experiment, and germination rates were not significantly different among any of the ferulic acid concentration tested (Figure 1). Hyphal elongation was inhibited at all concentrations of ferulic acid tested for the duration of the experiment (Figure 1). Hyphal lengths for all ferulic acid treatments were

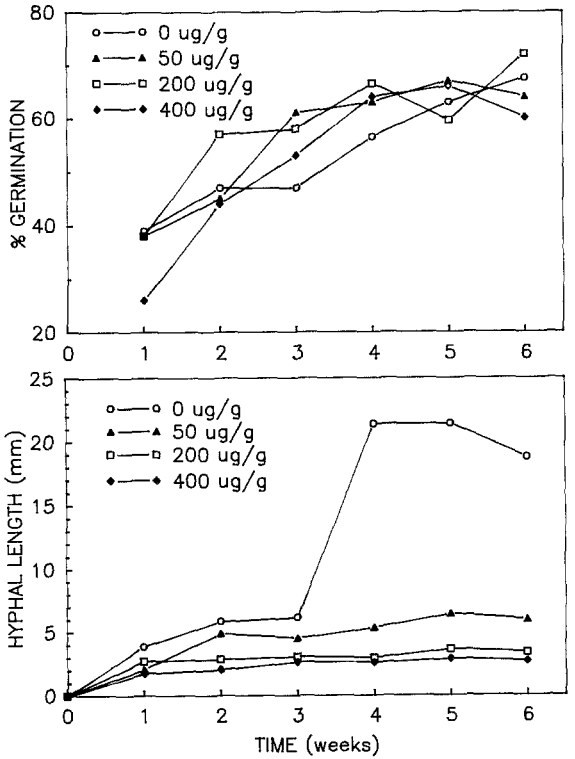

FIG. 1. Germination and hyphal elongation of *Glomus fasciculatum* spores subjected to increasing concentrations of ferulic acid in clover root exudate/Hoagland's medium over a six-week period.

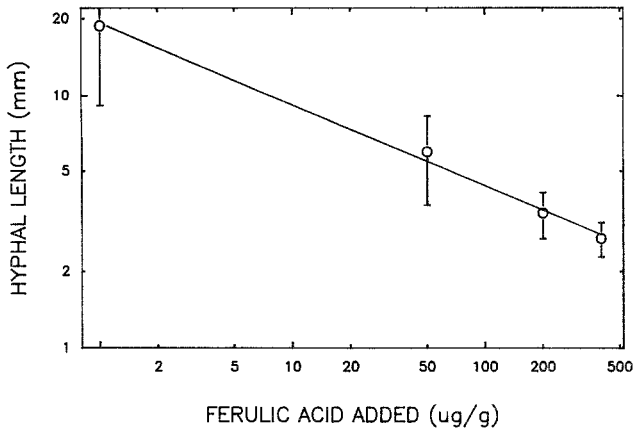

FIG. 2. Hyphal lengths of *Glumus fasciculatum* spores subjected to increasing concentrations of ferulic acid after six weeks. Data were log-log transformed and analyzed by linear regression. Values for r (-0.2) and P (0.002) are for the best-fit line from this transformation ($y = 0.52 - 0.09x$).

significantly less than the control from the fourth week on and exhibited a log-linear relationship (Figure 2, sixth week data shown; log-log transformation: $y = 0.57 - 0.09x$, $r = -0.2$, $P < 0.002$).

Growth of Asparagus With and Without VAM. The colonization of asparagus roots by *G. fasciculatum* decreased significantly with increasing ferulic acid concentration ($r = -0.6$, $P = 0.004$; Figure 3). Growth of mycorrhizal asparagus also decreased with increasing ferulic acid concentration ($r = -0.4$, $P = 0.08$; Figure 3). There was a significant correlation between plant growth

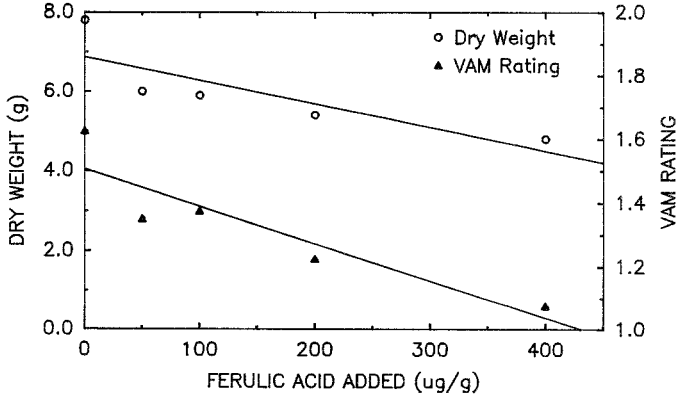

FIG. 3. Dry weights and VAM rating for mycorrhizal asparagus plants growing in a greenhouse and subjected to increasing concentrations of ferulic acid.

TABLE 1. TISSUE PHOSPHORUS LEVELS IN VAM AND NON-VAM ASPARAGUS PLANTS AT INCREASING CONCENTRATIONS OF FERULIC ACID ADDED TO SOIL IN THE GREENHOUSE

Ferulic acid added (μg/g)	Tissue P (% dry weight)[a]	
	Non-VAM	VAM
0	0.33	0.38
50	0.32	0.39
100	0.42	0.33
200	0.33	0.35
400	0.37	0.34

[a] ANOVA, NS

(dry weight) and VAM colonization ($P < 0.05$). There was no relationship between growth of nonmycorrhizal asparagus and ferulic acid concentration ($r = -0.01$, $P = 0.96$; data not shown). Growth of nonmycorrhizal asparagus plants was significantly greater than mycorrhizal asparagus plants at all ferulic acid concentrations ($P < 0.02$). Tissue P concentrations were not significantly different regardless of VAM treatment or ferulic acid concentration ($P > 0.50$, Table 1).

DISCUSSION

Ferulic acid inhibition of in vitro growth of *Glomus fasciculatum* was not due to a reduction in germination of chlamydospores, but instead to an inhibition in hyphal elongation. In the greenhouse, increasing ferulic acid concentrations led to a decrease in colonization of asparagus by *G. fasciculatum*. Ferulic acid could be affecting mycorrhizal colonization of asparagus either by restricting host–fungus contact, the fungus within the host root, or by limiting the formation of appressoria. Our results suggest that host–fungus contact may be affected: since ferulic acid inhibited hyphal elongation in vitro, then it may also be inhibiting fungal growth before root contact is established. However, under natural conditions, the effect of the toxin may be diminished by environmental or biotic factors. For instance, high soil moisture could facilitate leaching of the toxin from the soil or microbial alteration of the toxin may occur in the soil environment. Previous studies suggest that microbial degradation toxic compounds may occur. In these studies, asparagus residues were less toxic when subjected to an aerobic environment (Hartung, 1987).

The VAM-infected asparagus plants decreased in biomass with increasing

concentrations of ferulic acid. The decrease in biomass of asparagus plants probably is not due to a direct effect of ferulic acid on the plants since nonmycorrhizal asparagus growth was not altered by ferulic acid at any of the concentrations used. The reduced growth of mycorrhizal asparagus plants at higher ferulic acid concentrations may be associated with a reduction in the symbiotic effectiveness of *G. fasciculatum*. There is evidence for this in our results since the decline in mycorrhizal colonization was correlated to reductions in plant growth. Kiernan et al. (1983) found that growth of mycorrhizal sweetgum seedlings decreased with increasing fertilizer concentrations, while nonmycorrhizal plants were unaffected. This response to high fertilizer concentrations was apparently dependent on the VAM fungal species involved. In previous studies, we found that *G. fasciculatum* was capable of eliciting a positive growth response in asparagus (Wacker, 1988), so the reduced asparagus growth in the present study is not due to an incompatibility between symbionts. Although ferulic acid reduces fungal colonization, the fungus could still be depleting the host of carbohydrates. This could have led to the reduced plant growth that was evident at higher ferulic acid concentrations.

There was no apparent effect of ferulic acid on tissue P levels. It is possible that the fertilization schedule coupled with the relatively high soil moisture levels enabled soil P to be readily available to the plant and in high enough concentrations that ferulic acid did not affect uptake. High soil P levels can cause reductions in VAM colonization (Azcon et al., 1978; Black and Tinker, 1977; Graham and Menge, 1982). Even if soil P was at concentrations that inhibited VAM colonization, this would not have accounted for the gradual reduction in infection with increasing ferulic acid concentration, because all treatments received the same amount of added phosphorus. In both greenhouse and field studies, Wacker (1988) found that increased growth in mycorrhizal asparagus was not related to final tissue P concentrations.

The results of this study pose several questions regarding the role of VAM fungi in the growth of asparagus in the field. If allelochemicals can inhibit root colonization by VAM fungi, and if those fungi that infect the roots in the presence of these chemicals can depress growth, then asparagus growth could be debilitated through the loss of VAM-improved nutrient uptake as well as through the loss of carbohydrates to the fungus. Also, the production of allelochemicals may be involved in the succession of VAM fungi in asparagus fields (Wacker, 1988). Schenck et al. (1987) found a shift in VAM species occurring in coffee rhizospheres to *Acaulospora* spp. after four years. They suggested that shifts in species composition of VAM fungi may contribute to decline problems in perennial crops. Coffee produces allelochemicals and suffers from decline problems (Rice, 1984), as does asparagus. The possibility of allelochemical mediation of VAM fungal succession may be an important factor in decline situations. The effect of other allelochemicals on VAM fungi should also be

addressed. These allelochemicals could be acting either additively or synergistically in the inhibition of VAM colonization of asparagus roots. It is also possible that other root exudate compounds (Elias and Safir, 1987) could mask the effect of ferulic acid on mycorrhizal infection in asparagus.

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CHEMICALLY INDUCED METAMORPHOSIS OF POLYCHAETE LARVAE IN BOTH THE LABORATORY AND OCEAN ENVIRONMENT

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Abstract—Planktonic larvae of the marine polychaete *Phragmatopoma californica* preferentially attach to substrata and metamorphose to the adult form upon contact with cement in tubes built by conspecifics. This gregarious settlement and metamorphosis contributes to the formation of large aggregations or reefs. Larvae also metamorphose upon contact with 2,6-di-*tert*-butyl-4-methylphenol (DBMP), a possible aromatic analog of cross-linked dihydroxyphenylalanine (DOPA) residues (present in the polyphenolic protein cement as 2.6% of the amino acid residues). Morphogenesis occurs in the laboratory when larvae are exposed to DBMP either adsorbed to solid surfaces or when dissolved in dimethyl sulfoxide (DMSO) to render it soluble in seawater. Larvae in the ocean were induced to settle and metamorphose on plates coated with DBMP prior to their deployment in the ocean. This is the first report in which a defined organic molecule, identified as an inducer (or precursor to an inducer) of larval settlement and metamorphosis in the laboratory, has been shown to induce these processes in the ocean. Both forskolin and isobutylmethylxanthine (IBMX) induce metamorphosis of *P. californica* larvae, presumably by causing increases in intracellular cyclic AMP (cAMP). A discussion of the pathway controlling chemically mediated metamorphosis and evidence suggesting the possible role of cAMP in the process are presented. Other compounds known to increase intracellular cAMP levels, including arachidonic, linoleic, and palmitoleic acids, found by other workers to induce settlement and metamorphosis of *P. californica*, may exert this activity by direct modification of internal cAMP levels in the larvae.

Key Words—Polychaeta, larvae, planktonic, *Phragmatopoma californica*, Sabellariidae, metamorphosis, settlement, recruitment, chemoreception, 2,6-di-*tert*-butyl-4-methylphenol, 3,4-dihydroxyphenylalanine, cross-linking, sclerotization.

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INTRODUCTION

Unlike insects, which begin metamorphosis to the adult form on a timetable determined for the most part by changes in endogenous hormonal levels (Borrer et al., 1976), the planktonic larvae of many species of benthic marine invertebrates often delay metamorphosis to the adult form until triggered by external environmental cues. For insects, the dispersing adults generally possess the external receptors necessary for recognition of the appropriate habitat for the more sedentary larval stages. The reverse is true for many species of marine invertebrates, where the dispersing larvae are responsible for recognizing the habitat that is optimal for survival and reproduction of the more sedentary adult forms.

Detection of environmental conditions by many invertebrate larvae has been shown to involve the recognition of very specific chemical cues associated with the surfaces on which the organisms settle or attach (for reviews, see Crisp. 1974, 1984; Scheltema, 1974; Chia and Rice, 1978; Burke, 1983a, 1986; Hadfield, 1978, 1986; Rittschof and Bonaventura, 1986). For a variety of organisms, extracts prepared from substrata that induce metamorphosis of larvae upon contact have been shown to act as morphogens in solution. Although some information about the chemical nature of these inducers has been determined, their actual molecular structures remain largely unknown. Prior to the work reported here, there had been no defined organic molecule identified as an inducer of larval settlement and metamorphosis in the laboratory that could be demonstrated to induce these processes in the ocean.

Evidence suggests that chemically induced metamorphosis of many species of marine invertebrate larvae is under control of the larval nervous system with specific sensory receptors acting, at least in part, to interface the nervous system with the environment (Hadfield, 1978; Morse et al., 1979, 1980a,b; Burke, 1983a, 1984; Baloun and Morse, 1984; Morse, 1984, 1985; Morse and Morse, 1984; Trapido-Rosenthal and Morse, 1985, 1986a,b; Baxter and Morse, 1987). As reviewed by Morse (1985), several cases of chemically induced settlement and metamorphosis of marine invertebrate larvae involve neurotransmitter-like inducers. GABA (γ-aminobutyric acid), an important neurotransmitter in vertebrates and invertebrates, and GABA analogs produced by certain marine algae induce metamorphosis of several species of invertebrates, including 12 species of abalone and other gastropod mollusks. The nudibranch *Phestilla sibogae* undergoes metamorphosis in the presence of choline (Hadfield, 1984). DOPA (3,4-dihydroxyphenylalanine) and its analogs have been shown to induce metamorphosis of the mussel *Mytilus edulis* (Cooper, 1982) and the oyster *Crassostrea gigas* (Coon et al., 1985; Coon and Bonar, 1987). Burke (1984) has demonstrated that larvae of the sand dollar *Dendraster excentricus* partially metamorphose in response to catecholamines [as well as in response to

electrochemical stimulation of parts of the larval nervous system (Burke, 1983b)]. While it is likely that several of these cases involve activation at internal, synaptic, or hormonal receptors (Coon et al., 1989), recent evidence suggests that the GABA analogs and the algal GABA mimetic act at a specialized chemosensory receptor to induce *Haliotis* settlement and metamorphosis (Trapido-Rosenthal and Morse, 1986a,b; Morse and Trapido-Rosenthal, in preparation).

The marine polychaete *Phragmatopoma californica* forms large aggregations of tubes in which they live (Figure 1) along the Pacific Coast of North America. These aggregations are due, at least in part, to gregarious settlement of the larvae (Jensen and Morse, 1984). Wilson (1968, 1970a,b) demonstrated that the related sabellariid polychaetes, *Sabellaria alveolata* and *Sabellaria spinulosa* metamorphose on tubes built by conspecifics. Larvae of *Phragmatopoma californica* metamorphose into the adult form upon contact with tube material cemented together by conspecifics (Jensen and Morse, 1984). The adhesive material is comprised largely of a DOPA cross-linked protein that otherwise resembles silk in amino acid composition (Jensen and Morse, 1988). Of the component amino acids found in the adhesive, only the aromatic amino acids, especially DOPA, were observed to have metamorphosis-inducing activ-

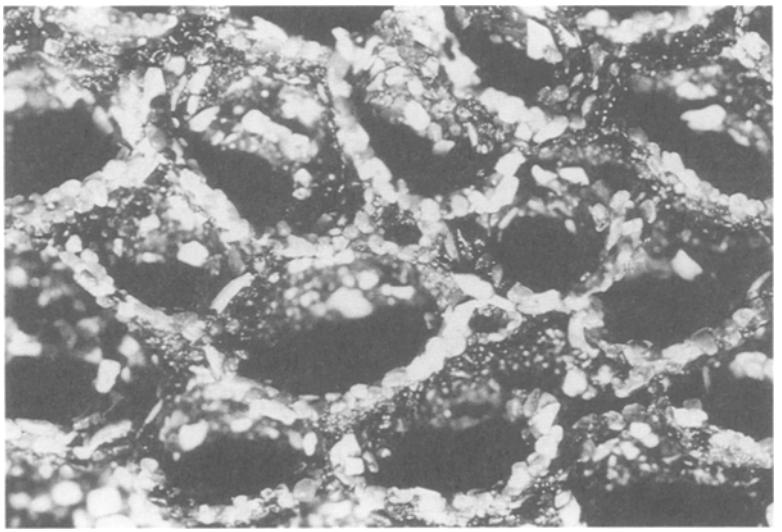

FIG. 1. Close-up photograph of part of an aggregation of *Phragmatopoma californica* formed in the field as a result of gregarious settlement. Shown are several apertures of tubes, each aperture representing a single genetically distinct individual. Photograph courtesy of Robert Sisson.

ity when exposed to larvae in the laboratory, albeit at a low level (10–15% at 10^{-5} M, with higher concentrations often resulting in incomplete metamorphosis, with evidence of toxicity) (Jensen, 1987; Jensen and Morse, unpublished observations).

In this paper we report the morphogenic activity of a possible analog of a cross-linked form of DOPA residues in the polyphenolic adhesive protein, the aromatic compound 2,6-di-*tert*-butyl-4-methylphenol (DBMP), on larvae of the marine polychaete *Phragmatopoma californica* when tested both in the laboratory and in the ocean. A discussion of the pathway controlling chemically mediated metamorphosis and evidence suggesting the possible role of cyclic AMP (cAMP) in the process are presented. Some of the data presented here were published in a doctoral dissertation (Jensen, 1987).

METHODS AND MATERIALS

Clumps of tubes containing adult *Phragmatopoma californica* were collected from intertidal and subtidal areas in Santa Barbara County, California. Both males and females were induced to release gametes by removing them from their tubes. Eggs were fertilized with a dilute sperm suspension to produce embryos. Resultant larvae were raised in the laboratory at the University of California, Santa Barbara, by the methods described by Jensen and Morse (1984) and Jensen (1987).

Tube material used in settlement experiments was obtained by allowing adults in the laboratory to cement glass beads (0.45–0.50 mm; Glasperlin; B. Braun Melsungen AG, obtained from Van Waters and Rogers, Los Angeles, California) onto the anterior ends of preexisting tubes. Pieces of this tube material then were broken into individual glass beads, which had worm-secreted cement on their surfaces (Jensen and Morse, 1984). Control glass beads were soaked in seawater for a comparable period of time (to allow a film of organic molecules and microorganisms to develop on the surfaces).

Because DBMP is not readily soluble in seawater, it was presented to larvae either (1) solubilized in dimethyl sulfoxide (DMSO) prior to addition to seawater (with appropriate DMSO controls) or (2) dissolved as a 0.1 M solution in HPLC-grade ethyl ether, which then was evaporated onto glass slides for laboratory experiments or onto settlement plates (described below) for use in the ocean.

In order to alter intracellular levels of cAMP, larvae were exposed to varying concentrations of forskolin (an activator of adenyl cyclase, the enzyme responsible for converting ATP to cAMP; Seamon et al., 1981) and to isobutylmethylxanthine (IBMX; an inhibitor of cAMP-specific phosphodiesterase, an enzyme responsible for the degradation of cAMP; Robinson et al., 1971). In

order to solubilize forskolin in seawater, it was dissolved first as a concentrated stock solution in DMSO. Controls containing the corresponding DMSO concentrations were tested with larvae. Additionally, larvae were exposed directly to the phosphodiesterase-resistant dibutyryl cAMP (and in a control treatment, the corresponding cGMP derivative) in seawater. The pH of all solutions ranged from 7.7 to 8.1. Chemicals were purchased from Sigma Chemical Company, except for HPLC-grade ether, which was purchased from Mallinkrodt Chemical Company.

For tests of induction of metamorphosis of *Phragmatopoma californica* larvae in the laboratory, multiple samples of five larvae each were incubated at 16°C in 10 ml seawater (which had been filtered to 5 μm and briefly exposed to ultraviolet radiation to kill bacteria) with or without a test chemical (or glass beads with or without cement) in replicate glass vials (diam. = 2.4 cm). The number of larvae metamorphosed in each vial was determined as a function of time by microscopic examination; n = number of replicate trials. Larvae were considered metamorphosed when they had shed their provisional setae and rotated their tentacles forward (see Eckelbarger, 1977, for a complete description of metamorphosis); this marks the termination of their swimming stage and the beginning of morphogenesis to the benthic adult stage.

Field work on settlement and metamorphosis was conducted in the subtidal zone near the Goleta Pier at Goleta Beach, California (latitude 34°25′N, longitude 119°49′W). Settlement plates were constructed from 1/8-in.-thick Plexiglas plastic with dimensions of 8 × 8 cm, with only the inner 5.5 × 5.5 cm analyzed for settlement. Plates were coated with Devcon underwater epoxy and 75 μl of a 0.1 M solution of DBMP in HPLC-grade ethyl ether was evaporated onto the appropriate test surfaces. Plates were attached, with the use of plastic cable ties through two drilled holes, to racks made of Chemgrate, a fiberglass-reinforced polyester grating. Plates were arranged in a random block design, with each rack containing a replicate of each treatment. The racks were attached horizontally in the subtidal zone by divers with the aid of SCUBA gear to rigid supports made of Unistrut, a fiberglass-reinforced polyester material. Settlement surfaces faced downwards at a distance of approximately 1 m from the bottom. The downward facing placement of settlement surfaces precluded the possibility of larval settlement to test surfaces resulting from any passive pharmacological narcotic or "knock-down" effect, but rather limited these studies to the measurement of active attachment of larvae to surfaces. Water depth at the study site averaged 6.5 m, varying with tidal height (maximum range 6.0–7.6 m). Plates were put into the field during the height of settlement season (Jensen and Morse, in preparation) on March 5, 1986, and retrieved seven days later. After return to the laboratory, plates were analyzed with the use of a dissecting microscope for settled organisms living in tubes which they built.

Additionally, smaller (2 × 2 cm) replica plates, made of the same materials (with equivalent amounts of DBMP coated onto the surfaces) as those put in the field, were tested for morphogenetic activity with larvae cultured in the laboratory. Larvae were exposed to plates (with and without DBMP on the surfaces) in 400 ml of seawater (that had been filtered to 5 μm and briefly UV irradiated) at 16°C in the dark. The numbers of larvae attached and metamorphosed on the plates were determined upon microscopic examination at 18 hr.

To determine if the morphogenetic activity of DBMP remained on the plates after prolonged immersion in the ocean, the following experiment was conducted. Plates similar to those described above (with and without DBMP applied to the surfaces) were constructed and placed in the subtidal zone at Naples Reef, 11.5 km from the Goleta Beach study site. There was no settlement and metamorphosis of *Phragmatopoma californica* on study plates in this area; therefore, no conspecific cement or other gregarious cues could have been added to the surfaces. Plates were left in the field for five days, then kept in the laboratory in gently flowing seawater for a further three days. Additionally, a new plate with DBMP freshly applied to its surface was constructed in the laboratory. Several small scrapings from all three plate types were removed with a scalpel and tested for morphogenetic activity. The scrapings were placed into three separate glass vials (diam. = 2.4 cm), each containing 10 *P. californica* larvae in 10 ml seawater (16°C, filtered to 5 μm, briefly UV irradiated). At 24 hr, the number of larvae metamorphosed in each vial was determined.

RESULTS

Glass beads, which had been used by adults in tube building and which contained adherent plaques of the DOPA cross-linked cement (Jensen and Morse, 1984, 1988), induced 95% of the larvae to settle and metamorphose within 18 hr of exposure (Figure 2). No larvae metamorphosed in either control treatments (seawater alone, or filmed glass bead control), even after 110 hr of exposure.

DBMP, a chemical that forms oxidative cross-links and intermediates potentially similar to those which may form during the enzymatically catalyzed oxidative cross-linking of DOPA residues in proteins (Figure 3), was a potent inducer of metamorphosis of larvae in the laboratory, both in the solubilized form and as a deposit coated on glass slides. DBMP induced 100% metamorphosis of larvae when tested at a concentration of 10^{-5} M (in DMSO, in seawater) in the experiment shown in Figure 4. Although the observed response was always concentration dependent, the apparent threshold concentration for induction varied between experiments (data not shown). At concentrations higher than 10^{-4} M, DBMP was increasingly toxic to larvae.

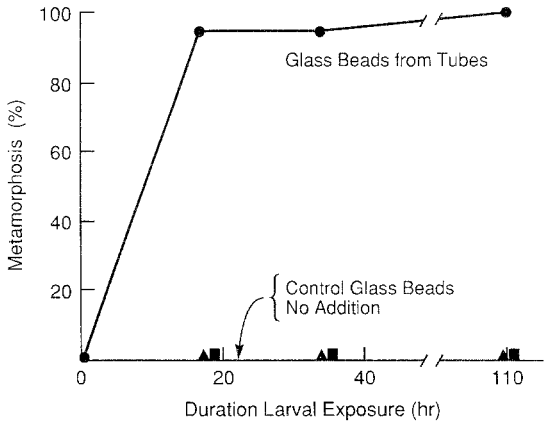

FIG. 2. Glass beads (with cement), from tubes constructed by adults in the laboratory, induce metamorphosis of conspecific larvae. Data are expressed as mean percentage of larvae metamorphosed per vial (five larvae tested per vial) at indicated times of exposure. All SD = 0, except glass beads from tubes at 17 hr and 34 hr, SD = 10%. Larvae 40 days postfertilization; $n = 4$.

When larvae contacted glass slides coated with DBMP (20 μl of a 0.1 M concentration in ether evaporated onto 1-cm-diam. circular slides), they responded immediately in a manner similar to the behavioral response seen when larvae encounter native tube material. They alternately contracted along the left and right axes, resulting in a lateral twisting motion; and they protruded their abdominal setae, which are characteristic of the adult tube dwelling form. They vigorously stroked their tentacles and head region along the substratum; metamorphosis generally followed. When assayed at 8 hr, 80% of the larvae had metamorphosed on slides coated with DBMP (Figure 5). Even at 56 hr, no larvae had metamorphosed in either control (ether alone evaporated onto slides, or seawater controls). Higher doses of DBMP were increasingly toxic to larvae, and even at the tested dose there appeared to be some slight interference with tube-building or adhesion.

In the field, settlement and metamorphosis of *Phragmatopoma californica* larvae were enhanced by the presence of DBMP on plate surfaces (Table 1). All plates treated with DBMP had significantly higher settlement than control plates in both the field and laboratory ($P < 0.02$, one-way ANOVA, data log-transformed for homogeneity of variances; Sokal and Rohlf, 1969). In a separate experiment (data now shown), it was demonstrated that the effect of DBMP on the plate surfaces in the field was not dose-dependent above the lowest dose applied.

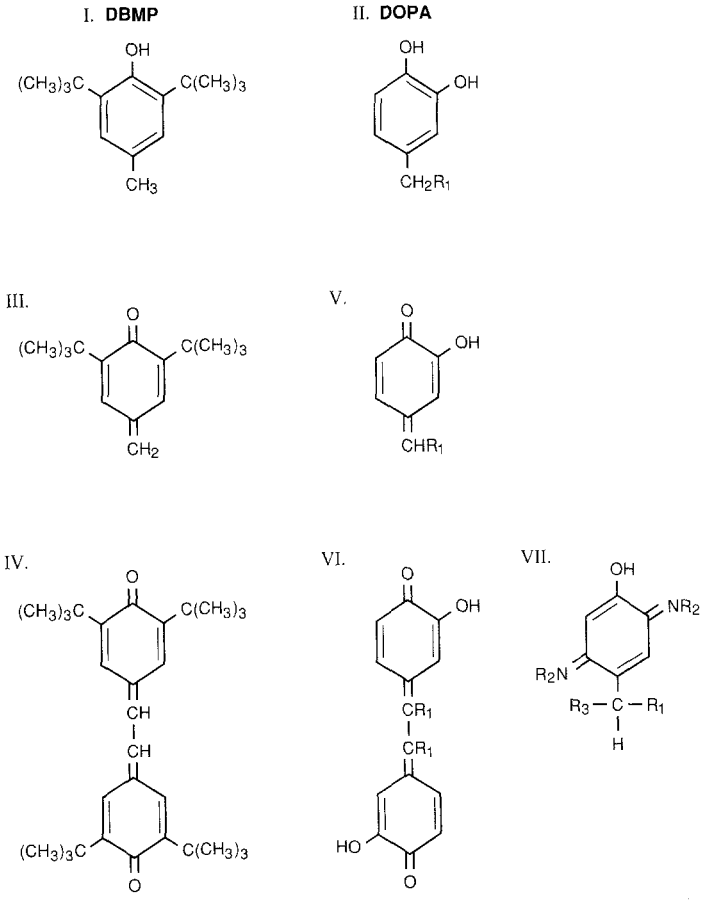

FIG. 3. Structures of DBMP (I, 2,6-di-*tert*-butyl-4-methylphenol) and a DOPA (II, 3,4-dihydroxyphenylalanine) residue in a polyphenolic protein. Representative oxidation products (of an entire family of possible products; Lamarre et al., 1981; and other references in text) of DBMP are III, a quinone methide 2,6-di-*tert*-butyl-*p*-quinomethane, and IV, a dimer, 2,6,2′,6′,tetra-*tert*-butyl stilbene-quinone. V, hypothetical quinone methide suggested by Sugumaran and Lipke (1983) to be an intermediate during the polyphenol oxidase-catalyzed sclerotization of insect cuticle. VI, one possible hypothetical sclerotized product formed by the cross-linking of two DOPA-derived quinone methide molecules in a manner analogous to the dimerization of two DBMP-derived quinone methide molecules in the formation of a stilbene-quinone. VII, hypothetical catechol cross-linked protein (modified from Lipke et al., 1983, Figure 34, p. 73). R_1 indicates [CH-(peptide backbone)] for the DOPA peptides; R_2 indicates proteins added by ring substitution; R_3 indicates protein added by β-sclerotization.

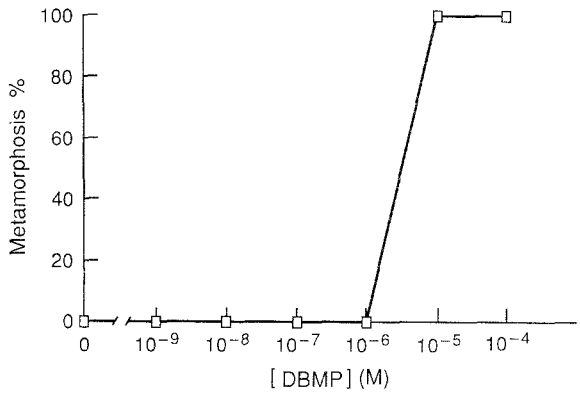

FIG. 4. Concentration-dependent metamorphic response of *Phragmatopoma californica* larvae to DBMP after 21 hr of exposure. All concentrations of DBMP (including 0) contained 0.1% (v/v) DMSO to solubilize the DBMP. Data are expressed as mean percentage of larvae metamorphosed per vial (five larvae tested per vial). All SD = 0. Larvae 42 days postfertilization; $n = 2$.

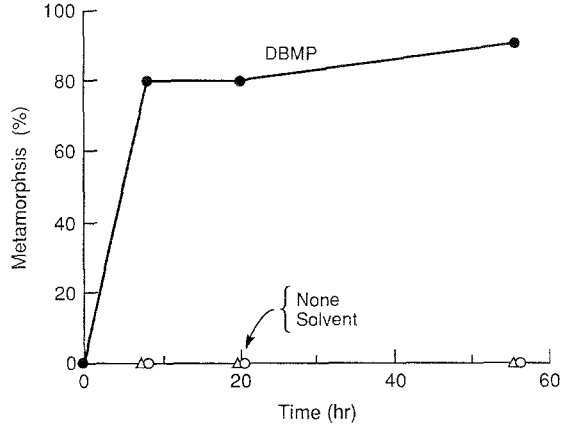

FIG. 5. Metamorphosis of *Phragmatopoma californica* larvae on glass slides coated with DBMP (20 μl of 0.1 M DBMP in ether) or ether alone (as a control). Additional control vials contained no glass slides. Data are expressed as mean percentage of larvae metamorphosed per vial (five larvae tested per vial) at the indicated times of exposure. All SD = 0, except DBMP coated plates at 56 hr SD = 14%. Larvae 36 days postfertilization; $n = 2$.

TABLE 1. METAMORPHIC RESPONSE OF *Phragmatopoma californica* LARVAE TO SURFACES COATED WITH DBMP IN THE LABORATORY AND OCEAN[a]

	Laboratory		Ocean	
Inducer	$\bar{X}$	SD	$\bar{X}$	SD
None	0.25	0.50	0.83	1.65
DBMP	4.00	1.83	9.09	7.33

[a] Data are expressed as mean and standard deviation of the numbers metamorphosed per unit area (4.0 cm^2 for laboratory plates; 100 cm^2 for ocean plates). $n = 4$ for all treatments.

Plates coated with DBMP maintained their morphogenic activity after eight days (five days in the field, followed by three days in the laboratory) of exposure to seawater (Table 2). All larvae metamorphosed after incubation with either newly applied DBMP on epoxy or that which had been in the ocean. No larvae metamorphosed on scrapings from control plates exposed to seawater for the same time period.

Addition of both forskolin (an activator of adenyl cyclase, an enzyme responsible for converting ATP to cAMP) and IBMX (an inhibitor of cAMP-specific phosphodiesterase, an enzyme responsible for the degradation of cAMP) induced settlement and metamorphosis of *Phragmatopoma californica* larvae in a concentration-dependent manner (Figure 6). Neither dibutyryl cAMP nor dibutyryl cGMP (both tested at concentrations of 5×10^{-3}, 10^{-3}, 5×10^{-4}, and 10^{-4} M) induced settlement and metamorphosis of larvae; the permeability of these compounds was not determined.

TABLE 2. PERSISTENCE OF MORPHOGENIC ACTIVITY OF DBMP-COATED PLATES AFTER 8-DAY INCUBATION IN SEAWATER (5 DAYS IN SUBTIDAL ZONE OF OCEAN, FOLLOWED BY 3 DAYS IN GENTLY FLOWING SEAWATER IN THE LABORATORY.)[a]

DBMP	Incubated in seawater	*Phragmatopoma* metamorphosis (%)
Yes	No	100
Yes	Yes	100
No	Yes	0

[a] To test the persistence of the morphogenic effect of DBMP on plates in seawater, scrapings were taken from plates that had been in seawater for 8 days with and without DBMP on surfaces and from a newly constructed plate with DBMP on its surface. Scrapings were put into vials containing 10 ml seawater and 10 *P. californica* larvae. The percentage of larvae metamorphosed was determined in each vial after 24 hr.

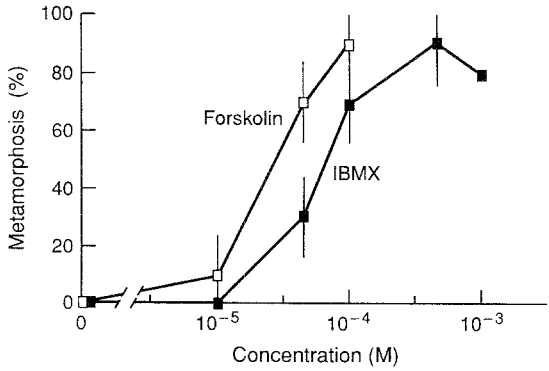

FIG. 6. Metamorphic response of larval *Phragmatopoma californica* to IBMX and forskolin after 24 hr of exposure. Forskolin was solubilized in DMSO before it was added to seawater. The DMSO in all forskolin treatments was adjusted to 0.3% (v/v). Seawater controls with and without DMSO showed no metamorphic response. Data are expressed as mean and standard deviation of the percentage of larvae metamorphosed per vial (five larvae tested per vial). Larvae 46 days postfertilization; $n = 2$ for all treatments.

DISCUSSION

Cessation of the planktonic stage of larvae of the marine polychaete *Phragmatopoma californica* and the subsequent metamorphosis to the sedentary adult form are critical events in the animal's life cycle. During the six or more weeks of development to the stage where larvae are competent to metamorphose (Dales, 1952; Eckelbarger, 1977; and results from cultivation of larvae in our laboratory), the potential is great for dispersal both to new settlement sites and away from sites favorable for adult growth and reproduction. Natural selection for a larva that is able to distinguish a favorable adult habitat is increased by the fact that, unlike some other species of polychaetes, postmetamorphic animals are not capable of leaving their tubes to start new ones (Hartman, 1944; Jensen and Morse, unpublished data). The commitment to settle and metamorphose on a particular substratum, therefore, is permanent. As seen in Figure 2, larvae metamorphosed specifically on beads that had adherent plaques of bioadhesive cement applied by adults during tube-building.

The behavior of animals in the laboratory does not always reflect accurately the behavior of animals in the field where other variables are many, uncontrollable, and may produce unpredictable, interactive effects. Although the induction of settlement and metamorphosis of larvae of many species of marine invertebrates has been shown in the laboratory to depend upon very specific external cues, the actual contribution of these factors to settlement in the field is poorly understood. For instance, Butman et al. (1988) recently dem-

onstrated that larvae of *Capitella* sp. and of *Mercenaria mercenaria*, when in still water, metamorphosed in response to cues from organic-rich muddy sediments or clean glass beads, respectively, but only *Capitella* exhibited enhanced metamorphosis in response to the same materials in moving water. Cuomo (1985) demonstrated that sulfide acts as an enhancer of larval settlement for *Capitella* larvae in both the laboratory and seminatural conditions. The work reported here is the first instance in which a defined organic molecule identified as an inducer (or precursor to an inducer; see below) of larval settlement and metamorphosis in the laboratory has been demonstrated to induce these processes in the ocean. The results reported here on the enhanced attachment and metamorphosis by *Phragmatopoma californica* larvae to plates coated with DBMP (Table 1), and those of Cuomo (1985), demonstrate that larvae of some species do respond to the chemical nature of substrata during settlement and metamorphosis.

Prior work has shown that larvae of *Phragmatopoma californica* can delay metamorphosis for periods of several months in the absence of contact with newly built conspecific tube (Jensen and Morse, 1984). Chemical analysis of the adhesive secreted by *P. californica* revealed that the bulk of the adhesive is proteinaceous (Jensen and Morse, 1988). The amino acid composition of this adhesive resembles that of silk, with the short-chain amino acids (glycine, serine, and alanine) accounting for 60% of the total residues (Jensen and Morse, 1988). The material is highly insoluble in a variety of solvents and detergents (Jensen and Morse, unpublished data), apparently due to extensive cross-linking of DOPA residues (present in the polymeric cement as 2.6% of the residues) and possibly lysine (present as 12% of the residues) (Jensen and Morse, 1988).

When larvae are incubated with any of the individual amino acids identified in the tube cement (less than 1% of amino acid residues remain unidentified) at concentrations ranging from 10^{-6} to 10^{-3} M, the aromatic amino acids (especially DOPA) were observed to show evidence of some metamorphosis-inducing activity, but at low levels often resulting in abnormal metamorphosis with evidence of toxicity (Jensen, 1987; Jensen and Morse, unpublished observations). A variety of aromatic compounds induce larvae to undergo premetamorphic search behavior or metamorphosis, but results are often variable and show evidence of toxicity often resulting in incomplete metamorphosis. The susceptibility of free DOPA and its analogs to oxidation in seawater complicates these analyses. However, the properties of tyrosine and DOPA residues within the adhesive polypeptide chain, and the properties of these residues after undergoing enzyme-catalyzed formation of specific oxidation products and cross-links, are very different from those of the free amino acids (cf. Waite, 1987).

DBMP reacts with oxygen under alkaline conditions; many of the less active oxidation products, including dimers and intermediates, have been iden-

tified (Figure 3; cf. Cook, 1953; Metro, 1955; Kharasch and Joshi, 1957; Neureiter, 1963; Lichtenthaler and Ranfelt, 1978; Foley and Kimmerle, 1979; Spitz, 1980; Lamarre et al., 1981; Thompson et al., 1989). Shown in Figure 3 are the structures of DBMP (I) and a DOPA residue in a polyphenolic protein (II) and various products, either known or hypothetical, formed from each. Two representative compounds formed from the oxidation of DBMP, a quinone methide (2-6-di-*tert*-butyl-*p*-quinomethane) (III), and a dimer (2,6,2′,6′-tetra-*tert*-butyl stilbene-quinone) (IV) are illustrated in Figure 3. For the sake of brevity, only these two oxidation products of DBMP are shown in Figure 3, although Lamarre et al. (1981) identified 11 oxidation products of DBMP (three of which were dimers) through high-performance liquid chromatography (HPLC), and an entire family of oxidation and/or cross-linked products can be postulated. Larvae actually may be responding to any of these compounds that could be formed on the plates in seawater.

Although no detailed structures of cross-links formed by DOPA-containing proteins have been purified and characterized, several types of enzyme-catalyzed cross-linking interactions are possible (Waite, 1987). Some of the compounds involved in insect cuticle sclerotization by catechols have been identified (see Andersen, 1977, 1979; Andersen and Roepstorff, 1981; Lipke et al., 1983; Schaefer et al., 1987). Cross-linking between catechols and proteins mainly can occur either through ring substitutions or by covalent linkage at the β carbon, adjacent to the aromatic ring (β-sclerotization). A phenolic dimer, presumably a product of β-sclerotization, has been isolated and characterized from insect cuticle (Andersen and Roepstorff, 1981; Roepstorff and Andersen, 1981). Evidence suggests that the β-sclerotization catalyzed by polyphenol oxidase proceeds through the formation of a quinone methide intermediate (structure V, Figure 3) (Sugumaran and Lipke, 1983; Sugumaran, 1987; Sugumaran and Semensi, 1987). In Figure 3, structure VI is a hypothetical DOPA residue cross-link of the stilbene-quinone type, which might be formed between polypeptides from the quinone methide by a process analogous to that characterized for DBMP. Many other cross-links involving DOPA residues are possible. Structure VII is a hypothetical mixed cross-linked species, also derived from the reactive quinone methide intermediate, as modified from Lipke et al. (1983). Thus far, no DOPA-containing protein cross-link structure has been unambiguously resolved. For mosquito cuticle, the ratios of cross-linking by ring substitution to cross-linking via β-sclerotization varies both between species and between life stages (Sugumaran and Semensi, 1987).

DMBP induces both premetamorphic search behavior characteristic of larval contact with the native tube-associated inducer and complete metamorphosis. DBMP is likely to form cross-links in the aqueous environment, and at least one of the cross-linked structures, or other oxidation products, such as a quinone methide, may approximate a structure attributed to DOPA residue cross-

links (or intermediates in their formation) in adhesive proteins (cf. Andersen and Roepstorff, 1981; Lipke and Sugumaran, 1983; Waite, 1987; Figure 3). The existence of a quinone methide intermediate compound in sclerotization in arthropod cuticles is fairly well established (Sugumaran, 1983; Lipke et al. 1983; Sugumaran et al., 1987), and such a structure may be present in cross-linked DOPA proteins as well (Rzepecki and Waite, personal communication).

At present, however, the mechanism for induction of metamorphosis remains speculative. DBMP is an antioxidant that has been shown to protect stressed membranes from damage, apparently a result of its inhibitory effect both on lipid peroxidation (Koster and Slee, 1983; Koster et al., 1983; Girotti et al., 1986; Deuticke et al., 1987) and on oxidative protein damage (Deuticke et al., 1987). DBMP also has been shown to increase membrane fluidity (Singer and Wan, 1977). These and other possible effects on membrane activity or intracellular activity cannot be ruled out in terms of mechanism for induction of metamorphosis.

Clearly, however, the inductive effect by DBMP on *Phragmatopoma californica* larvae is not simply a pharmacological narcotic or "knock-down" effect, as demonstrated by the fact that the chemically coated plates tested in the ocean (Table 1) were downward-facing. Larvae actually "settled" upward onto these surfaces. Additionally, upon contact with DBMP, larvae immediately undergo the behavioral response associated with premetamorphic "search" behavior on native-tube inducer. Also, there is some species specificity of the response; DBMP-coated surfaces had no apparent effect on abalone (*Haliotis rufescens*) larvae that were developmentally competent to metamorphose (Jensen and Morse, unpublished data). The nature of the active inducer derived from DBMP, and its possible relation to the natural inducers of settlement and metamorphosis in *P. californica* is a subject of our current investigations.

It has been demonstrated that certain free fatty acids, including palmitoleic, linoleic, and arachidonic acids, induce settlement and metamorphosis of *Phragmatopoma californica* larvae (Pawlik, 1986; Pawlik and Faulkner, 1986). Pawlik (1986) extracted and concentrated a mixture of free fatty acids that induced metamorphosis of *P. californica* larvae from clumps of tubes, collected from the field, which likely contained contaminating biological material. It is possible that these free fatty acids constitute a second class of compounds in the tube material that induce larval metamorphosis under natural conditions. However, native tubes contain a variety of organic material, including algae, foraminiferans, mollusk shells, ostracods, and even fecal pellets. Fatty acids are common in marine organisms in their conjugated forms and may have been extracted from biological material contaminating the tubes. Whether they are present on the surface of the tubes where larval contact is possible or whether they were extracted from other organic material incorporated in the tube is uncertain. Whether they act at specific larval receptors or whether they act else-

where, for instance, in a manner similar to potassium induction by altering membrane ion conductance (Yool et al., 1986), by altering cAMP responses (see below), or by modifying receptor sites through detergent-like action on membranes, increasing membrane fluidity, or possibly other unknown mechanisms (cf. Baumgold, 1980) also is unknown. If they are present in the actual tube cement, they are there only in small amounts, as demonstrated by analyses of morphogenic tube material constructed from inert glass beads (Jensen and Morse, 1988).[1]

Both forskolin and IBMX induced metamorphosis of *Phragmatopoma californica* larvae in a concentration-dependent manner. Forskolin acts to increase intracellular cAMP by activating the enzyme adenyl cyclase, which converts ATP to cAMP (Seamon et al., 1981). IBMX inhibits cAMP specific phosphodiesterase, an enzyme that causes degradation of cAMP, thus having an overall effect of increasing intracellular cAMP (Robison et al., 1971). However, the exact sites of action by both of these agents are not clearly defined (Seamon and Daly, 1985; Cary and Mendelsohn, 1987), and other sites of action may be possible. Free fatty acids, including arachidonic, linoleic, and palmitoleic acids, are known to alter cyclic AMP levels in a wide variety of systems. They have been demonstrated to have a stimulatory effect of the basal level on cAMP in mammalian cerebral cortex, possibly by acting on the lipid domain of the membrane associated with adenyl cyclase (Baba et al., 1984). Free fatty acids also have been found to elevate cAMP production in a membrane system controlled physiologically by catecholamines (Orly and Schramm, 1975). In view of the data presented here, indicating that cAMP may be involved in the control of metamorphosis in *Phragmatopoma*, it is possible that the free fatty acids shown to induce metamorphosis of *P. californica* (Pawlik and Faulkner, 1986) may be doing so simply by acting on adenyl cyclase activity. Dibutyryl cAMP did not induce metamorphosis, but its permeability was not tested with *P. californica* larvae, which are likely to possess a cuticle similar to that of *P. lapidosa* (Eckelbarger, 1978); such a cuticle would be expected to be relatively impermeable to the negatively charged nucleotides.

It has been shown that an increase in the concentration of K^+ in defined seawater medium induces settlement and metamorphosis of *Phragmatopoma californica* larvae, presumably by causing the depolarization of externally accessible, excitable cells (Yool et al., 1986). These results implicate ionic depolarization and possibly cAMP in the metamorphic process, suggesting involvement of the larval neural system in the induction of metamorphosis by

[1] Recent evidence further indicates that free fatty acids are not responsible for the natural gregarious attachment and metamorphosis of *Phragmatopoma californica* larvae on conspecific tubes. Free fatty acids are not found in clean preparations of the natural inducer; a low level can be extracted from biologically contaminated worm masses, apparently present as a result of degradation of lipids during extraction procedures (Jensen and Morse, submitted).

the native tube cement, although their possible roles and dependence upon receptor activitation await resolution by further neurophysiological and in vitro analyses. It appears likely that the inducing chemical component(s) of native tube cement (and possibly DBMP) interact with receptors at the cell surface, which in turn activate adenyl cyclase to convert ATP to cAMP, a second messenger. This could act indirectly to open ion channels, causing excitatory depolarization of the membrane, initiating the cascade of behavioral and morphogenic events occurring during metamorphosis.

Tufts of sensory cilia on tentacles of larvae of *Phragmatopoma lapidosa* have been implicated in substratum selection (Eckelbarger and Chia, 1976). Similar ciliary tufts, also presumably sensory in function, have been described for *Sabellaria cementarium* (Smith and Chia, 1985) and for *P. californica* (Amieva et al., 1987). Upon encountering native tube-cement or DMBP-coated surfaces, *P. californica* larvae increase contact with substrata by rubbing their tentacles, mouth, and head region against them. It is likely that chemosensory cells are located primarily in these regions.

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ALLELOPATHY OF SMALL EVERLASTING (*Antennaria microphylla*) Phytotoxicity to Leafy Spurge (*Euphorbia esula*) in Tissue Culture

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Abstract—Media and media extracts from callus cultures of small everlasting (*Antennaria microphylla*) inhibited leafy spurge (*Euphorbia esula* L.) callus tissue and suspension culture growth (50 and 70% of control, respectively) and were phytotoxic in lettuce and leafy spurge root elongation bioassays (64 and 77% of control, respectively). Hydroquinone, a phytotoxic compound previously isolated from small everlasting, was also biosynthesized by callus and suspension cultures of this species. Exogenously supplied hydroquinone (0.5 mM) was toxic to leafy spurge suspension culture cells and was only partially biotransformed to its nontoxic water-soluble monoglucoside, arbutin, by these cells. This report confirms the chronic involvement of hydroquinone in the allelopathic interaction between small everlasting and leafy spurge.

Key Words—Allelopathy, *Antennaria microphylla*, small everlasting, *Euphorbia esula*, leafy spurge, tissue culture, hydroquinone, arbutin, glucosyltransferase, biotransformation.

INTRODUCTION

The rapid invasion of the perennial weed leafy spurge (*Euphorbia esula* L.) into the rangelands of the upper Great Plains of the United States has resulted in significant losses to livestock production. This noxious weed is toxic to rangeland cattle (Selleck et al., 1962) and phytotoxic to palatable forage species

(Selleck, 1972). Although it is suppressed by cultivation and/or chemical methods, leafy spurge cannot be eradicated (Lym and Messersmith, 1983). The high cost of control of this noxious weed by traditional methods has prompted the evaluation of biological control agents as an alternative method of control (Pemberton, 1985).

Dense stands of the deep-rooted leafy spurge were observed to be susceptible to encroachment by small everlasting (*Antennaria microphylla*), a low-growing, shallow-rooted perennial (Selleck, 1972). This susceptibility could not be attributed solely to competitive factors. The dichotomous morphological character of the two plants and experimental observations provided evidence that the growth inhibition of leafy spurge in these stands was due to natural chemical phytotoxins (allelochemicals) produced by small everlasting. Three biologically active phenolic compounds, 1,4-dihydroxybenzene (hydroquinone), hydroquinone β-D-glucopyranoside (arbutin), and 3,4-dihydroxycinnamic acid (caffeic acid), identified from ether extracts of dried small everlasting plant material, were shown to inhibit leafy spurge seed germination and radicle elongation at physiological concentrations (Manners and Galitz, 1985). Hydroquinone was considered to be the most important component of the allelopathic interaction of these two species in the field.

Allelochemicals may be leached from plants as water-soluble nontoxic derivatives that undergo chemical alteration once they reach the soil matrix or the target plant (Balke et al., 1987). It is difficult to study the mechanisms of such allelopathic interactions in the field because of complicating abiotic and microbiological factors. This study describes the first in vitro examination of allelopathic intraplant interactions utilizing both the phytotoxic and the target species in tissue culture. This system was employed to examine specific biochemical processes associated with the allelopathy of small everlasting toward leafy spurge without the inherent complications of field or whole-plant studies. Tissue cultures of the two species were utilized to (1) determine the susceptibility of both target (leafy spurge) and nontarget (lettuce) species to the bioactive compound(s) produced by small everlasting and (2) test the ability of small everlasting to biosynthesize and exude phytotoxic secondary metabolites in culture.

METHODS AND MATERIALS

Cell Cultures. Leafy spurge cell suspension cultures (Accession No. ND 101) established from stem tissue at the Bioscience Research Laboratory in Fargo, North Dakota, were grown in the dark (28°C) on a New Brunswick rotary shaker (100 rpm). Small everlasting cell suspension cultures were established from leaf-derived callus cultures and were grown under identical condi-

tions. Cells were maintained by weekly subculture on Gamborg's B5 medium (Gamborg et al., 1968) containing 1 ppm (w/v) (2,4-dichlorophenoxy)acetic acid (B5 + 1). The inoculation rate was 5 ml of mature (7-day-old) culture to 50 ml of B5 + 1 medium in foam-plugged Erlenmeyer flasks (250 ml).

Callus Cultures. Callus cultures of small everlasting were established from leaf strips (approximately 1 cm in length) cut from field-collected plants and sterilized (1% sodium hypochlorite, 20 min). The culture medium used for callus initiation was Murashige and Skoog's medium (Murashige and Skoog, 1962) amended with 0.4 ppm (w/v) (2,4-dichlorophenoxy)acetic acid, 0.4 ppm (w/v) naphthalene acetic acid, and 0.2 ppm (w/v) kinetin (6-furfurylaminopurine). After three months, calli derived from these tissues were transferred to and maintained on B5 + 1 medium. All callus culture media contained 0.8% agar, were adjusted to pH 5.6, and were autoclaved for 20 min (121°C).

Leafy spurge callus cultures derived from sterilized root sections (approximately 1 cm in length) were established on B5 + 0.4 ppm (w/v) (2,4-dichlorophenoxy)acetic acid. After the first subculture, they were transferred to and maintained on B5 + 1 medium (28°C, dark) with subcultures performed every four weeks.

Cocultures. Preweighed pieces of callus tissue from both species (one week from the last subculture) were allowed to grow under treatment conditions for one week (28°C, dark) before reweighing. Data are expressed as percent increase in gram fresh weight per week. The same experiments were performed in bisected Petri dishes such that the calli grew in the same dish but on separate media.

Cell Culture Bioassay. Test hydroquinone solutions were prepared in absolute methanol and aseptically introduced into the flasks of leafy spurge suspension culture cells at a final methanol concentration of 1% or less (Davis et al., 1978). Inoculations were performed 24 hr after subculture to fresh medium. Cells were harvested seven days after inoculation (vacuum filtration onto Miracloth) and then washed with distilled water. Fresh weight determinations were made after air had been pulled through the filtered cells for 2 min.

Cells were steeped in 95% ethanol (1 hr, 80°C) and filtered. The filtrate was evaporated (in vacuo) to dryness, redissolved in methanol (2 ml), and injected (10 μl) onto a C18 reverse-phase HPLC column (Waters Z-module cartridge; H_2O–MeOH, 75:25 v/v; 2 ml/min; UV detection). Arbutin, hydroquinone (retention times 2.45 and 3.23 min, respectively; 280 nm), and benzoquinone (retention time 4.54 min; 254 nm) were quantitated by comparison with authentic standards. Liquid media from the filtered cells were placed in a separatory funnel and extracted with ether. The ether layer was back-extracted (aqueous $NaHCO_3$, 10%), and the resulting ether layer was analyzed for hydroquinone (UV; λ_{max} = 294 nm) using an HP 8451A Diode Array spectrophotometer.

Lettuce Seed Germination Bioassay. A lettuce seed germination bioassay was used to corroborate the presence of leachable phytotoxin(s) in the media on which the calli were growing. Fifty sterilized lettuce seeds (var. Burpee Greenhart) were placed on the agar medium in each test jar once the callus had been removed. Data were collected after 48 hr of dark incubation (28°C). Germination was defined as protruding radicles at least 2 mm in length.

Media Extract Bioassay. Test solutions were dissolved in 9-cm Petri dishes containing hot water agar (30 ml). When the plates had cooled, 10 pregerminated seedlings (radicles 2 mm long) of lettuce or leafy spurge were placed on the surface. Increases in radicle length were measured after 48 hr (28°C, dark).

Media Extraction and Fractionation. After three to four weeks of growth, small everlasting callus tissue was removed from its agar medium, and the latter was continuously extracted with methanol for 24 hr (Soxhlet). The resulting extract was concentrated to 2 ml (in vacuo) and loaded onto an Amberlite XAD-7 column (100 × 12 mm). Several bed volumes of distilled water were passed through the column followed by methanol. Ten methanol fractions (2 ml each) were collected and used in lettuce and leafy spurge root elongation bioassays.

Statistical Analysis. All bioassays included three replications. Individual determinations of germination, root length, and cell or callus culture weight were averaged and subjected to a one-way analysis of variance. F values were calculated to determine significant differences between treatments at the 0.05% level.

RESULTS AND DISCUSSION

Coculturing of comparable amounts of small everlasting and leafy spurge callus tissue on the same medium showed a significant inhibition of leafy spurge callus growth without significant inhibition of small everlasting callus growth (Table 1). A similar inhibition of spurge callus growth was observed when it was grown on medium on which small everlasting callus had been grown previously and then removed. A comparable experiment with calli grown in bisected Petri dishes produced no inhibitory effect of small everlasting callus on leafy spurge callus until the leafy spurge callus was transferred to the portion of the media on which small everlasting was cultured (data not shown). These observations demonstrate the ability of small everlasting to produce nonvolatile phytotoxic secondary metabolites in culture.

The coculturing experiments were corroborated in a lettuce seed germination bioassay. It was observed that the germination of sterile lettuce seeds was reduced by 81% when placed on nutrient agar media from which small everlasting callus had been removed. A similar experiment using medium on which leafy spurge callus had been grown produced a much smaller reduction (15%) in lettuce seed germination.

TABLE 1. COCULTURING EXPERIMENTS WITH CALLUS CULTURES OF SMALL EVERLASTING AND LEAFY SPURGE

Treatment	Increase (%) in gram fresh wt.[a]
I. Leafy spurge control	27.21[b]
II. Leafy spurge, cocultured with small everlasting	13.84[c]
III Leafy spurge on agar from which small everlasting was removed after 1 week	14.25[c]
IV. Small everlasting, cocultured with leafy spurge	31.45[b]
V. Small everlasting control	27.70[b]

[a] After one week of growth at 28°C in 24 hr darkness.
[b] The controls are not significantly different from each other, or from treatment IV.
[c] Significantly different from the leafy spurge control at the 0.05% level.

Root elongation bioassays of Amerberlite XAD-7-fractionated small everlasting media extracts showed bioactivity in only one fraction (lettuce root, 64% of control; leafy spurge root 77% of control). This fraction also reduced overall fresh weight in a suspension culture of leafy spurge by 30%. Experimental results confirm that bioactive compound(s) derived from small everlasting cultures can leach from the tissue and accumulate in the growth media (Gould and Murashige, 1985). The observed phytotoxicity of small everlasting callus and the inhibition of lettuce seed germination and leafy spurge and lettuce seedling root elongation by small everlasting media extracts corroborate previous whole-plant experiments and suggest that the phytotoxic effect is of chemical origin. Attempts currently are underway to isolate and characterize these phytotoxins from the media on which small everlasting has been growing.

Hydroquinone was detected in extracts of small everlasting callus. The occurrence of hydroquinone is consistent with the participation of this phytotoxin in the allelopathic interaction between small everlasting and leafy spurge and the suggestion that the mechanism of this interaction is based on differential metabolism of hydroquinone (Manners and Galitz, 1985; Manners, 1987). Increasing concentrations of hydroquinone introduced aseptically into cell culture flasks inhibited the growth of leafy spurge suspension culture cells during logarithmic and linear growth stages (Table 2). Growth inhibition was less dramatic after the spurge cells reached the stationary growth stage (days 12 and 16) and a concentration of 1×10^{-3} M hydroquinone was required to show acute inhibition of growth below the controls over the 16-day growth period. Cells treated with all but the highest concentration (1×10^{-2} M) were able to absorb hydroquinone from the media. A concentration of 5×10^{-4} M was used to evaluate hydroquinone as a chronic allelochemical and, at this concentration, a sublethal phytotoxic response was produced during all stages of cell growth

TABLE 2. EFFECT OF INCREASING CONCENTRATIONS OF HYDROQUINONE ON GROWTH OF LEAFY SPURGE SUSPENSION CULTURE CELLS[a]

Treatment	Fresh weight (g)			
	Day 4	Day 8	Day 12	Day 16
1% Methanol control	10.92[b]	19.61[b]	24.90[b]	24.55[b]
10^{-5} M Hydroquinone	11.16[b]	15.90[b]	23.48[b]	24.09[b]
10^{-4} M Hydroquinone	7.35[b]	14.44[b]	23.93[b]	23.40[b]
10^{-3} M Hydroquinone	1.35[c]	1.46[c]	19.58[c]	18.20[c]
10^{-2} M Hydroquinone	cells dead			

[a] Ether extractions of the media from the above samples showed no traces of hydroquinone, with the exception of the 10^{-2} M treatment.
[b] Not significantly different from the control for the same harvest date at the 0.05 level. Data are the average of three experiments (three treatment replications per experiment).
[c] Significantly different from the control for the same harvest date at the 0.05 level. Data are the average of three experiments (three treatment replications per experiment).

(Figure 1). A comparable experiment with arbutin showed no reduction in growth of leafy spurge cells at any of the test concentrations (data not shown).

The absorption of hydroquinone by leafy spurge cells over a broad range of concentrations suggested that the cells detoxified hydroquinone via gluco-

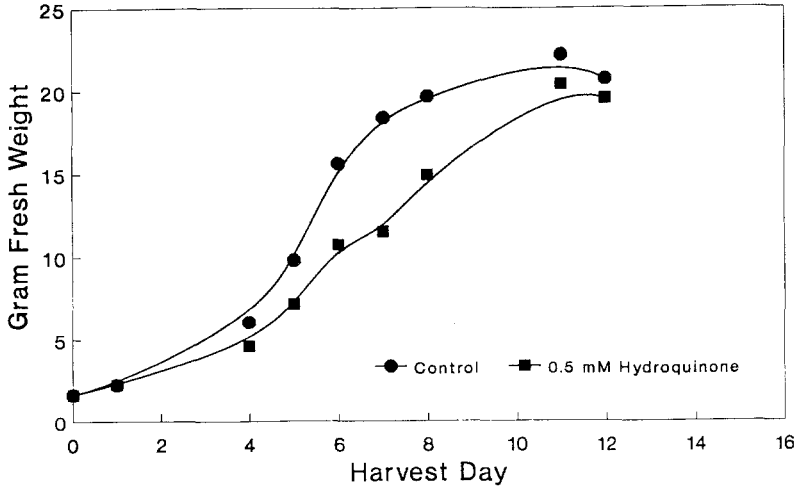

FIG. 1. The effect of 0.5 mM hydroquinone on the growth of leafy spurge suspension culture cells. Data points represent the average of three experiments (three replications per harvest day).

sylation to form arbutin. High-pressure liquid chromatographic analysis of extracts from leafy spurge suspension culture cells treated with sublethal concentrations of hydroquinone (Table 3) showed that cells treated once with 5×10^{-4} M hydroquinone converted 85% of this compound to arbutin during a short exposure time (six days). A higher sublethal concentration of hydroquinone (1×10^{-3} M) was also partially detoxified. Arbutin levels in the cells treated with 1×10^{-3} M hydroquinone increased, but the efficiency of glucosylation decreased to 71% and growth inhibition was more severe. Enzyme assays with cell-free extracts of treated leafy spurge suspension culture cells confirmed the presence of a UDPG-dependent glucosyltransferase capable of catalyzing this reaction. Glucosyltransferase activity was also measured in cell-free extracts of small everlasting callus tissue; however, the enzyme has not been purified to homogeneity in either species (Hogan and Manners, unpublished data).

It is apparent that both small everlasting and leafy spurge utilize a UDPG-dependent glucosyltransferase enzyme (EC 2.4.1) to conjugate a glucose moiety to hydroquinone, producing the more water-soluble, less phytotoxic glucoside, arbutin. Glucosyltransferases that detoxify allelochemicals are probably inducible, i.e., synthesized de novo in the presence of the aglycone (Balke et al., 1987). Preliminary glucosyltransferase activity measurements in cell-free extracts show leafy spurge to be less efficient in the detoxification of hydroquinone than small everlasting (Hogan and Manners, unpublished data). Data indicate that as the arbutin pool increases in leafy spurge tissue culture cells, biotransformation rates are diminished. Continuous exposure of leafy spurge to physiological concentrations of hydroquinone in the field over a long period of time could produce chronic growth inhibition. This observation is consistent with a proposed hydrolysis–equilibrium mechanism (Manners and Galitz, 1985),

TABLE 3. DETECTION OF ARBUTIN IN HYDROQUINONE-TREATED LEAFY SPURGE SUSPENSION CULTURE CELLS

Treatment	Fresh wt.[a]	arbutin (μg)/g fresh wt cells[b]	Glucosylation (%) of hydroquinone
Water control	18.39	0	0
Methanol control	18.35	0	0
5×10^{-4} M HQ[c]	12.67	17.65	85%
1×10^{-3} M HQ[c]	10.46	36.13	71%

[a] Fresh weight was measured seven days after inoculation.
[b] Arbutin was chromatographed on a C18 reverse-phase HPLC column using UV detection (280 nm).
[c] Hydroquinone treatments were added to the cells 24 hr after inoculation.

where the equilibrium of the detoxification reaction (glucosylation) may be shifted to favor the reverse reaction (hydrolysis) because of an increase in the concentration of arbutin. Differences in the kinetics of this glucosyltransferase enzyme may explain the differential toxicity of hydroquinone in the two species at sublethal (chronic) levels.

The differential ability of forage plants and noxious weeds to detoxify allelopathic compounds may provide a basis for selective weed control strategies not dependent on petrochemical-based herbicides (Jimenez-Osornio and Gliessman, 1987). Genetic manipulation of anabolic or catabolic pathways could favorably alter in vivo levels of allelochemicals. Such alterations could enhance natural competitiveness in the desired forage species at the expense of noxious weeds. Understanding the biochemical mechanisms of allelopathic interactions is a necessary prerequisite to the development of such weed control strategies. Results from these experiments emphasize the advantages of using tissue culture to study these interactions at a cellular–molecular level.

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RESPONSE OF POCKET GOPHERS (*Thomomys talpoides*) TO AN OPERATIONAL APPLICATION OF SYNTHETIC SEMIOCHEMICALS OF STOAT (*Mustela erminea*)

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Abstract—This paper reports on laboratory bioassays and a large-scale field trial of synthetic mustelid anal-gland compounds in controlled-release devices designed for operational application to burrow systems of northern pocket gophers (*Thomomys talpoides*). The field study was conducted in an apple orchard in the Okanagan Valley of British Columbia. In laboratory bioassays, a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane from the stoat (*Mustela erminea*) and 3,3-dimethyl-1,2-dithiolane from the ferret (*M. putorius*), dispensed in clay pellets (activated alumina), produced a significant avoidance response by gophers. All resident gophers were permanently removed from 4-ha control and treatment blocks prior to placement of stoat odor in burrows. As indexed by soil mounds, significantly fewer gophers colonized the treatment (40) than the control (68) removal area during a 5.5-month overwinter experiment. There was also a significant difference when comparing the number of mounds between pre- (79) and posttreatment (40) censuses. In addition, most gopher activity tended to occur on the perimeter of the treatment block. Abundance of gophers showed little difference between additional 4-ha control and treatment blocks where gophers had not been removed. The results of this study provide an alternative technique to toxicants for pocket gopher control on forest and agricultural land.

Key Words—Pocket gopher, avoidance response, mustelids, 2-propylthietane, 3-propyl-1,2-dithiolane, 3,3-dimethyl-1,2-dithiolane, anal-gland com-

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pounds, operational application, *Thomomys talpoides*, *Mustela* spp., controlled-release devices.

INTRODUCTION

The role of mammalian semiochemicals in wildlife management and crop protection has received considerable attention in recent years. There are several species of small mammals that are pests because of their feeding damage to agricultural and forest crops. Synthetic compounds from mustelid anal-gland secretions have produced significant avoidance responses in voles (*Microtus* spp.) (Sullivan et al., 1988a) and pocket gophers (*Thomomys talpoides*) (Sullivan et al., 1988b) in small-scale field trials. Earlier field studies also reported on significant suppression of feeding by snowshoe hares (*Lepus americanus*) when exposed to synthetic mustelid anal-gland compounds (Sullivan and Crump 1984) and volatile constituents of red fox (*Vulpes vulpes*) urine (Sullivan and Crump 1986a).

One of the most persistent pests in tree fruit orchards and forest plantations of the Pacific Northwest of North America is the pocket gopher (Barnes, 1973; Crouch, 1982; Anderson and Kluge, 1986; Sullivan et al., 1987). Although Sullivan and Crump (1986b) and Sullivan et al. (1988b) reported significant avoidance responses by pocket gophers to mustelid anal-gland compounds on areas < 1 ha, it still needs to be demonstrated that these predator odor semiochemicals will be effective over a larger area. A suitable controlled-release device for dispensing these compounds in gopher burrow systems is clearly an essential requirement for operational applications.

This paper reports on laboratory bioassay testing of mustelid anal-gland compounds in release devices designed for large-scale application to gopher burrow-systems and on an assessment of the effectiveness of stoat (*Mustela erminea*) anal-gland compounds dispensed on an operational basis in an apple orchard.

METHODS AND MATERIALS

Mustelid Anal-Gland Compounds. Anal-gland compounds from the stoat (2-propylthietane and 3-propyl-1,2-dithiolane) and the ferret (*M. putorius*) (3,3-dimethyl-1,2-dithiolane) were prepared according to Crump (1978, 1980a,b, 1982). 2-Propylthietane and 3-propyl-1,2-dithiolane were mixed in a 1 : 1 ratio. All compounds were dispensed in clay pellets (activated alumina, Cat. No. H026-00-050, Edwards High Vacuum, Oakville, Ontario, Canada), which ranged in diameter from 3 to 5 mm. One or two drops (depending on size of pellet) of each compound or mixture was applied by a 1-ml microsyringe to

each pellet and allowed to dry for 1 hr prior to laboratory trials. A mixture of 20 g of 2-propylthietane and 20 g of 3-propyl-1,2-dithiolane was applied by the above method on 5200 pellets (ca. 7.7 mg/pellet) for the field trial.

Laboratory Bioassays. The avoidance behavior of pocket gophers to the stoat mixture and ferret compound was assessed by tallying the number of individuals "captured" in control and treatment rooms of a Plexiglas bioassay arena. This apparatus and the bioassay procedure are essentially the same as those described in Sullivan and Crump (1986b). The arena had a Longworth live-trap extending into an opening in each of the control and treatment rooms, which simulated a burrow system for a gopher to enter. Five pellets with either 3,3-dimethyl-1,2-dithiolane or the 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane were placed in a plastic cap (2 cm in diameter) with a perforated cover and attached with adhesive tape to the floor of the tunnel of the live-trap in the treatment room. This procedure allowed the odor to penetrate the treatment room without contaminating the tunnel or fur of pocket gophers who might pass over the pellets when entering the trap. An empty plastic cap was attached in a similar manner to the trap in the control room.

Two groups of gophers were tested. Each group was tested initially with both arena rooms acting as controls to determine if there was a preference by individual gophers to enter one room or another. Control and treatment rooms were alternated for each trial, with individual gophers being tested only once per trial. Only one trial was conducted on a given day. The number of gophers "captured" in each room was tallied to assess the impact of the single compound or mixture on avoidance behavior. All gophers were collected from intensive study areas at Summerland and Vernon, British Columbia, Canada. Males and females were pooled for all trials. Sample sizes of each group varied, depending on number surviving throughout the trial period and exclusion from results of those individuals responding to neither control nor treatment conditions.

Field Trial. Four 4-ha blocks were established in a MacIntosh apple orchard (*Malus domestica*) at the Coldstream Ranch, 12 km east of Vernon, British Columbia. This 80-ha orchard was 30–35 years old with tree spacing varying from 4.7 × 6.2 m to 4.7 × 9.2 m. All blocks were located in areas that had particularly high gopher activity as reflected by fresh mounds of soil from excavation of burrows. Two "removal" blocks (A, control; and B, treatment) were located at opposite ends of the orchard. Two "nonremoval" blocks (C, control; and D, treatment) were situated at least 100 m from the removal areas so as to avoid any effect of gopher removal on population processes in the nonremoval areas.

Pocket gopher populations may be indexed by counts of fresh mounds of soil on the surface (Beck and Hansen, 1966; Reid et al., 1966). The number of fresh mounds on every second panel (between tree rows) was counted on each

block on November 3, 1987. During the period November 4–11, 1987, all gophers were permanently removed by live-trapping on the two removal blocks. Totals of 47 and 44 gophers were removed from blocks A and B, respectively. This removal aspect of the study simulated the operational use of toxicants as a means of gopher control. It also was a logical step for testing this repellent system after the results of Sullivan and Crump (1986b) and Sullivan et al. (1988b). These latter studies reported that removal of the target population prior to placement of repellent yielded better results than when gophers occupied the treatment area. No pocket gophers were removed from blocks C and D.

Clay pellets containing the 1 : 1 ratio of 2-propylthietane and 3-propyl-1,2-dithiolane were systematically placed in gopher burrows during November 4–11 (conducted immediately after gopher removal on block B). Each treatment block was organized into 144 squares (four 1-ha blocks of 36 squares each), and two sets of nine pellets each were placed in each square for a total of 2592 pellets per treatment block (B and D). Gopher burrows were located by probing the ground near fresh mounds and placing each set of nine pellets in the burrow and then covering the opening with soil to reseal the burrow system. This process acted to essentially "fumigate" the burrow systems with the synthetic odor of stoat.

After the overwinter period of 1987–1988, the number of fresh mounds on every second panel was counted on each block on April 24, 1988.

Statistical Analysis. Comparisons of the number of gophers captured in control and treatment rooms in the laboratory bioassays as well as number of mounds on control and treatment blocks in the field trial were analyzed by chi-square with significance levels of $P < 0.05$ and $P < 0.01$.

RESULTS

Laboratory Bioassays. Responses of pocket gophers to mustelid anal-gland compounds in clay pellets are illustrated in Figure 1. The control trials showed no difference in gopher preference for either arena room nor was there a difference between males and females. Gophers clearly avoided the mixture of 2-propylthietane and 3-propyl-1,2-dithiolane as well as the 3,3-dimethyl-1,2-dithiolane. The response of sample group B to the mixture was not significant ($P = 0.10$).

Field Trial. The responses of gophers to the stoat odor repellent on the removal and nonremoval blocks in the field trial are illustrated in Figure 2. As indexed by mounds, significantly fewer gophers colonized the treatment (40) than control (68) removal area during the 5.5-month overwinter experiment. This difference was also significant when comparing the number of mounds between the pre- (79) and postreatment (40) censuses. In addition, the majority

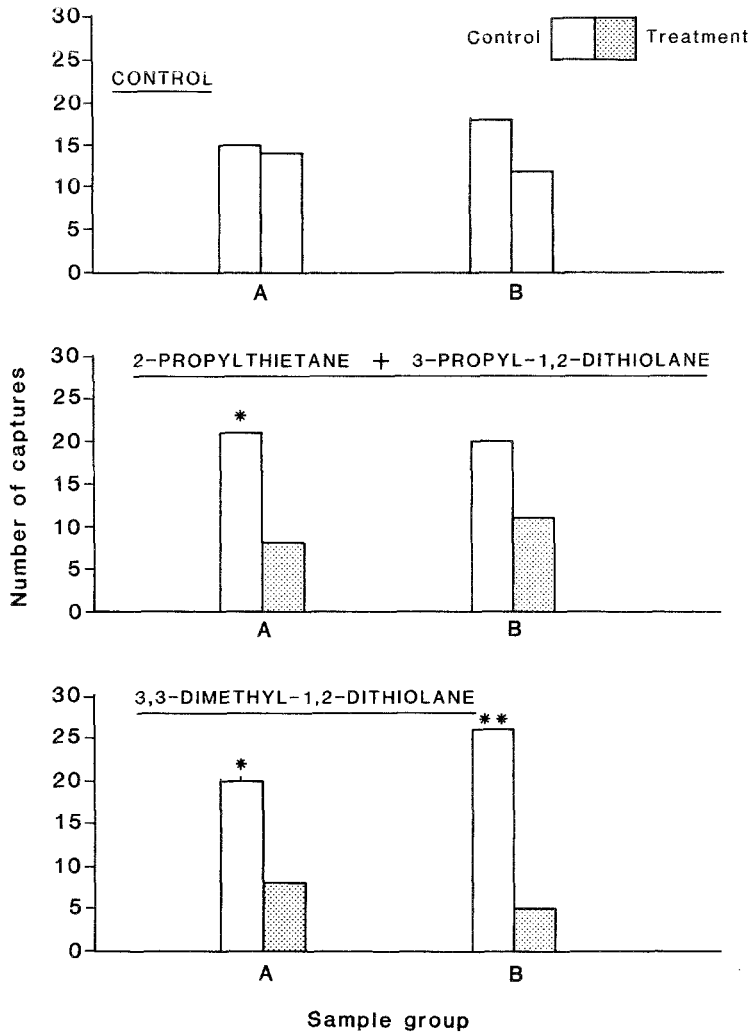

FIG. 1. Avoidance behavior responses ("capture in live-traps") of pocket gophers to mustelid anal-gland compounds on clay pellet release devices. $^{**}P < 0.01$; $^{*}P < 0.05$; significant difference by chi-square.

of mounds were observed to occur near the perimeter of the treatment block compared with a more uniform distribution on the control.

There was little difference in the number of gophers occupying the control and treatment blocks on the nonremoval areas. Fewer mounds were censused on the treatment (38) than control (54) in April, but this difference was not statistically significant ($P = 0.10$).

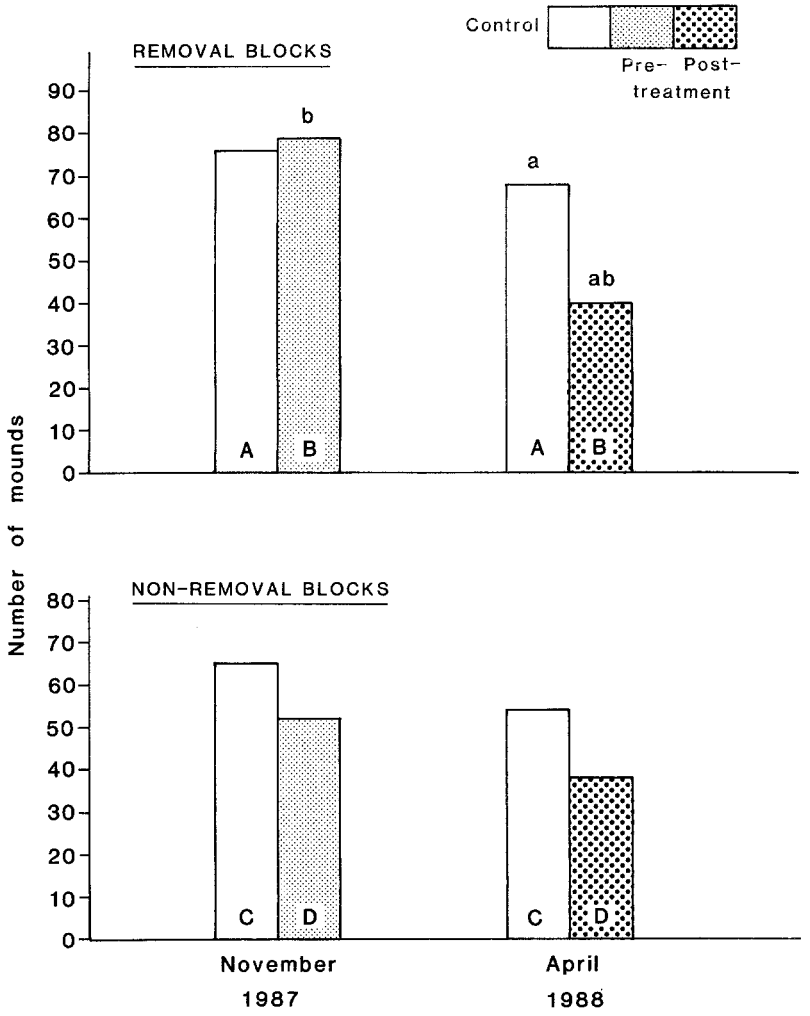

FIG. 2. Number of gopher mounds in field trial on control and treatment removal and nonremoval blocks. All gophers were removed from the control and pretreatment blocks (A and B) prior to operational application of stoat anal-gland compounds to burrow systems of the two treatment blocks (B and D). a-a, b-b, $P < 0.01$; significant difference by chi-square.

DISCUSSION

The results of our field trial, which was conducted in a practical orchard situation, demonstrated that pocket gophers would avoid synthetic stoat odor in an area where the original population of gophers was removed. This result was

similar to that reported by Sullivan and Crump (1986b) where, on nonremoval areas, the odor of mustelid anal-gland compounds could presumably not fumigate burrow systems because of the digging activities of resident gophers. In the subsequent study by Sullivan et al. (1988b), the distribution of gophers was limited to the perimeter of treated areas, but the density of animals was little changed between control and treatment areas. The present study has clearly shown that the abundance of gophers can be significantly reduced by these stoat anal-gland compounds, when applied to a relatively large area. In addition, the observed concentration of gopher activity on the perimeter of the treatment removal block suggested a level of avoidance similar to that recorded by Sullivan et al. (1988b) on smaller ($\leq$1 ha) study areas.

Although not quantitatively assessed, gopher mounds on the treatment removal block occurred in clumps compared with a relatively uniform distribution of mounds on the control. It was difficult, if not impossible, to determine if one or more gophers was responsible for several mounds in a given area of the treatment removal block. Thus, it is possible that the approximate 50% reduction in gopher abundance from pre- to posttreatment censuses on the removal may be an underestimate, in terms of the actual number of individuals colonizing the treatment area. Intensive live-trapping and use of radio telemetry in future studies could determine the specific responses of individual gophers to stoat odor repellents in a field situation.

This study has clearly identified a type of release device that could be used for dispensing mustelid anal-gland compounds in gopher burrows. The clay pellets with the mixture of 2-propylthietane and 3-propyl-1,2-dithiolane, and 3,3-dimethyl-1,2-dithiolane, which produced significant avoidance responses in laboratory bioassays, were readily applied to gopher burrows in an operational application. Our technique was similar to that used in poison baiting where grains or pelletized baits are inserted in burrows in large-scale applications on forest and orchard lands (Barnes, 1973; Barnes et al., 1982; Anderson and Kluge, 1986; Crouch, 1986). Thus, this technique could be adapted to existing gopher control programs with little or no change in logistics. These release devices could also be adapted to mechanical application via a burrow builder, which makes artificial burrows and dispenses baits (or repellents) on both agricultural and forest lands (Ward and Hansen, 1962; Anderson and Kluge, 1986).

The "removal" aspect of this study simulated the use of toxicants to control pocket gopher populations. As illustrated in Figure 2, gophers readily colonized the control removal block. The problem of resiliency (population recovery) in gopher populations has indicated that only short-term (if any) substantial control has been achieved with toxicants or other means of depopulation (Crouch, 1986; Sullivan, 1986; Sullivan and Sullivan, unpublished). Our stoat odor repellent is an alternative method that has produced better results than continued use of toxicants in reducing gopher populations. As indicated by our

laboratory results and those of Sullivan and Crump (1986b) and Sullivan et al. (1988b), 3,3-dimethyl-1,2-dithiolane from the ferret also should be dispensed in suitable release devices on an operational basis.

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COMPARISON OF RELEASE DEVICES FOR STOAT (*Mustela erminea*) SEMIOCHEMICALS USED AS MONTANE VOLE (*Microtus montanus*) REPELLENTS

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Abstract—Fenced enclosures were used to simulate peak populations of montane voles (*Microtus montanus*) for field bioassays of a stoat (*Mustela erminea*) scent mixture in various controlled-release devices. A 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane was dispensed in capillary tubes, clay pellets (activated alumina), rubber septa, and plastic rope. Release devices were placed near, or attached to, young apple trees planted in blocks in two enclosures containing high populations of voles. The stoat scent mixture in rubber septa and plastic rope significantly reduced vole attack of trees compared with a control, pellets, and capillary tubes. In terms of complete girdling (tree mortality), this odor in septa and rope also significantly reduced feeding compared with the control, and the rubber septa formulation was more effective than either the pellets or capillary tubes. Fenced populations of voles may be used effectively to conduct field bioassays during low years in vole cycles. Variations of release devices (with this stoat scent mixture) based on rubber septa for broadcast application to depress vole population density and survival, and plastic rope attached to individual trees to generate an avoidance response, should be used for forest and agricultural crop protection.

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Key Words—Montane vole, stoat, release devices, 2-propylthietane, 3-propyl-1,2-dithiolane, crop protection, feeding suppression, rubber septa, capillary tubes, plastic rope, clay pellets, *Microtus montanus*, *Mustela erminea*.

INTRODUCTION

Several species of voles in the genus *Microtus* inflict feeding damage to various forest and agricultural crops in North America, Europe, and Asia. The impact of these herbivorous rodents on crop production has been reviewed by Hansson and Nilsson (1975), Green (1978), and Byers (1984, 1985). Vole populations tend to have cyclic fluctuations every two to five years in northern latitudes (Krebs and Myers, 1974). Feeding damage is positively correlated with high population densities of voles and with optimum habitat (grass and/or shrub cover) conditions.

The use of toxicants has been the major method to control voles. An alternative technique based on synthetic predator odors as area repellents has considerable potential for protecting forest and agricultural crops from vole attack. In particular, anal-gland compounds from the stoat (*Mustela erminea*) have suppressed feeding by voles and have reduced the population size of voles in field experiments (Sullivan et al., 1988a,b).

A major difficulty in conducting field bioassays has been the periodicity of vole population and damage outbreaks. An alternative means is required to assess the efficacy of predator odor formulations during years when voles are scarce. In addition, improved release systems are needed for optimum effectiveness for long-term field use on an operational basis. We used fenced enclosures to simulate peak populations of voles (*M. montanus*) for field bioassays and assessed various controlled-release devices for repellent formulations.

METHODS AND MATERIALS

Stoat Odor Compounds and Release Devices. Anal-gland compounds, 2-propylthietane and 3-propyl-1,2-dithiolane, from the stoat, were prepared according to Crump (1978, 1980, 1982). In an attempt to simulate the anal-gland secretion of the stoat and in accordance with the positive results reported by Sullivan et al. (1988a), the above compounds were mixed in a 1:1 ratio. Purity of synthesized compounds ranged from 95% to 98%.

This mixture was dispensed in 140-μl capillary tubes (75 × 1.5 mm), rubber septa (Wheaton serum bottle sleeve stoppers 13 mm ID × 20 mm OD), hollow plastic rope with an internal wire (20 cm in length), and in clay pellets (activated alumina—Catalog No. H026-00-050, Edwards High Vacuum, Oakville, Ontario, Canada), which ranged from 3 to 5 mm in diameter. Approxi-

mately 20 mg of the mixture was placed in a given capillary tube using a 1-ml syringe with 20-gauge needle. The plastic rope was also loaded in this manner, after which the open end was sealed with epoxy resin glue. Each septum was loaded by depositing 20 mg of the mixture, dissolved in 1 ml hexane (after Heath et al., 1986), into the cup of the large end. One or two drops (average of 7.7 mg) of the stoat mixture was applied by syringe to each clay pellet.

Vole Populations in Enclosures. Duplicate contiguous enclosures (each 30 × 40 m) constructed of 0.635-cm galvanized welded mesh fencing were established at the Agriculture Canada Research Station, Summerland, British Columbia, Canada. The mesh fencing was buried to a depth of 30 cm with 90 cm above ground fastened to 7.5-cm-diameter posts at 3.1-m intervals. This study area was located in old field grassland habitat composed mainly of crested wheatgrass (*Agropyron cristatum*), orchard grass (*Dactylis glomerata*), quackgrass (*A. repens*), bluegrass (*Poa* spp.), and smooth brome (*Bromus inermis*), with a minor abundance of several herbaceous annuals. The climate is semiarid, so irrigation was conducted at three-week intervals during summer months to maintain apple trees (see below) and a suitable habitat for voles.

After completion of the enclosures in early June 1987, voles were captured from surrounding areas and introduced into the fenced areas. Vole introductions from outside the enclosures, and recruitment of young animals from reproduction within the resident population, were designed to produce peak populations for bioassays during the overwinter damage period of 1987–1988. Vole populations were monitored by live-trapping on a checkerboard grid with 48 (4 × 12) trap stations located at 7.6-m intervals in each enclosure. Two Longworth live-traps were set at each station near recently used vole runways. Traps were baited with whole oats, and coarse brown cotton was supplied as bedding. In a given trapping period, traps were set on the afternoon of day 1, checked on the morning and afternoon of day 2 and morning of day 3, and then locked open. Trapping periods were conducted at three-week intervals from June to September 1987 and a final session eight weeks later in November. All voles captured were ear-tagged with serially numbered tags, sex and breeding condition noted, weighed on Pesola spring balances, and point of capture recorded. Population densities were enumerated by minimum number of voles known to be alive at each trapping period (Krebs, 1966).

Tree Bioassays. Each enclosure had eight rows of 2- or 3-year-old crabapple (*Malus* spp.) trees on seedling rootstock planted at a spacing of 3 × 4 m. Five blocks of approximately 50 trees each were randomly assigned treatments within the two enclosures. The capillary tubes and rubber septa were attached to trees in their respective blocks by plastic twist-ties at 5 cm above the base of the tree. The plastic rope was also attached to each tree in this manner with the internal wire. Three or four clay pellets were placed on the ground near the base of a given tree in that block. Trees in the control block had no release devices.

All treatments were installed on December 3, 1987. Vole feeding damage to trees (proportion attacked and proportion girdled) was checked on March 31, 1988, when the experiment was terminated. Voles were no longer feeding on apple trees as preferred alternative vegetation was available.

Statistical Analysis. Comparisons of the number of voles present in each enclosure, and number of trees undamaged and surviving in control and treatment blocks, were analyzed by chi-square with significance levels of $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

Vole Populations. The number of voles in each of the enclosures during June to November 1987 is illustrated in Figure 1. Density of voles was similar in both enclosures with average fall (September–November) values ranging from 271 to 517 voles/ha. This level of density was significantly higher than the 110–120/ha recorded by Sullivan et al. (1988b) in natural grassland on a nearby study area. Because of the inability to disperse, enclosed populations of voles tend to reach abnormally high densities before overgrazing the habitat and declining (Krebs et al., 1969; Boonstra and Krebs, 1977). Thus, the high densities in our enclosures would have exerted extreme feeding pressure on available food sources, particularly apple trees, during the overwinter period.

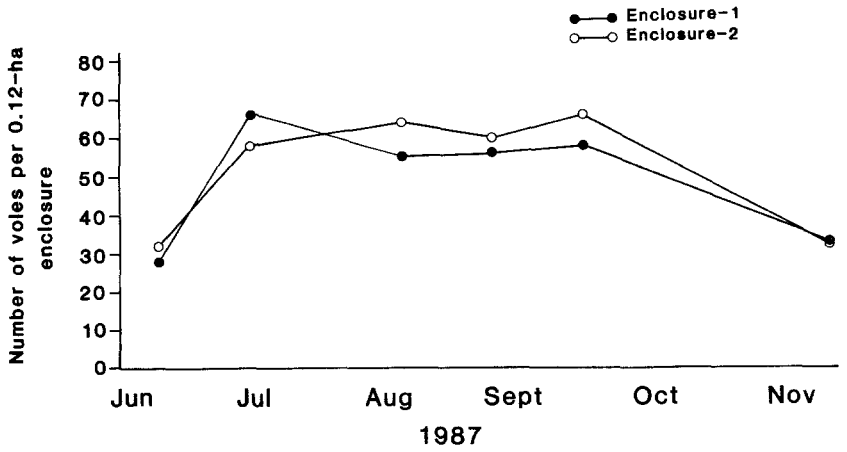

FIG. 1. Population densities (minimum number alive) of montane voles in each of the two enclosures from June to November 1987. Each enclosure represented 0.12 ha in area.

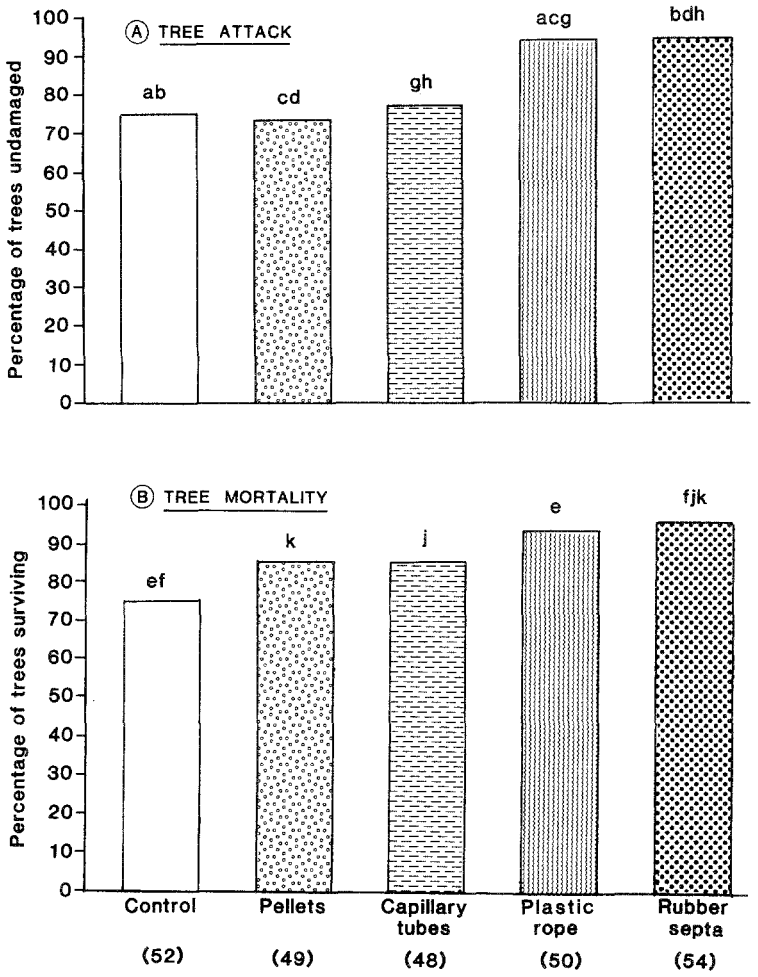

FIG. 2. Percentage of apple trees that were (A) undamaged by vole attack and (B) survived complete girdling by voles in control and treatment blocks. Tree sample size in parentheses. a-a, b-b, c-c, d-d, e-e, f-f, $P < 0.01$; g-g, h-h, $P < 0.05$; significant difference by chi-square. j-j, $P = 0.054$; k-k, $P = 0.06$.

Tree Bioassays. Results of the bioassay of stoat anal-gland compounds in different release devices are illustrated in Figure 2. The 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane, dispensed in rubber septa and plastic rope, significantly reduced vole attack of trees compared with the control, pellets, and capillary tubes (Figure 2A). In terms of complete girdling (tree mor-

tality), stoat odor released from rubber septa and plastic rope also significantly reduced feeding compared with the control (Figure 2B). In addition, the rubber septa formulation tended to be more effective than either the clay pellets or capillary tubes (Figure 2B).

These results were comparable to those reported by Sullivan et al. (1988a) for open field trials during a winter with peak populations of montane and meadow (*M. pennsylvanicus*) voles. The higher mortality (>80%) of control trees in Sullivan et al. (1988a) than recorded in this study was a function of tree size (circumference available for feeding attacks). One-year-old trees were used in the previous study and 2- to 3-year old trees in this study. In addition, the majority (69.2%) of control trees with intense feeding damage was recorded in the two tree rows furthest from an adjacent treatment block with stoat odor. These damaged trees were no longer standing, as all roots, bark, and vascular tissues of underground and basal stem parts had been eaten. This intensity of feeding was not recorded in other parts of the control block or in the treatment blocks. Thus, the degree of attack and mortality to control trees may have been greater if the treatment block had not been adjacent. However, it should be noted, from a practical point of view, that a 25% loss of young apple trees would clearly be a disastrous event for an orchardist.

The intensity of feeding pressure in our enclosures probably explains why capillary tubes were not an effective release mechanism for the stoat scent mixture compared with the earlier results of Sullivan et al. (1988a and b). Capillaries produce a point source of odor that may not be effective enough to generate a local avoidance (of the protected tree) response in a vole population outbreak. For example, the rubber septa opening that releases the odor was 8.1–8.7 times larger than that of the capillary tube. Similarly, the plastic rope presumably had the ideal area of release surrounding each tree with the repellent odor. The clay pellets distributed near the base of a given tree may not have provided enough of a concentration of stoat odor for voles to avoid approaching and feeding on the tree.

The relative release rates of 2-propylthietane and 3-propyl-1,2-dithiolane from each type of device were not measured in this study. However, this needs to be known before developing a suitable formulation for the rubber septa and plastic rope release devices. The rubber septa type of device may be most useful for broadcast application to reduce vole abundance in a forest plantation or agricultural environment as discussed by Sullivan et al. (1988b). Plastic rope, or a variation thereof, may provide optimal protection for individual trees or other crop plants.

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CALLING BEHAVIOR OF ALMOND MOTH (*Ephestia cautella*) FEMALES KEPT IN GLASS CAGES AND AIRBORNE PHEROMONE DEPOSITED ON GLASS SURFACES BY AIRSTREAM

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Abstract—The airborne pheromone emitted by calling almond moth (*Ephestia cautella*) females kept in individual glass cages was mainly adsorbed on the cage surface (60–65%), but sufficient pheromone was transferred by the airflow to the extended capillaries to be measured. Four calling positions of almond moth females in the glass cages were defined. The position in which the female faced the upwind flow and the gland was free to release the pheromone was the more commonly adopted position (39% of calling females). No significant difference was found in the blend proportion between capillary and cage washings in each calling position or between calling positions. The pheromone blend and amount emitted from each of five individual females was measured on three consecutive nights and both fluctuated during the 11 hr of collection, starting 3 hr before the onset of scotophase and continuing for 8 hr into it.

Key Words—Airborne pheromone, calling position, *Ephestia cautella*, Lepidoptera, pyralidae, (*Z*, *E*)-9,12-tetradecadienyl acetate, (*Z*)-9-tetradecenyl acetate, individual fluctuation, blend composition.

INTRODUCTION

A knowledge of the individual behavior of female and male insects in courtship and mating is important in understanding the preference for one specific mate over another (Lloyd, 1981). An important parameter in this behavior is the individual blend composition of the sex pheromone released by either the female or the male to attract its mate. It has been previously shown that females of the

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almond moth (*Ephestia cautella*) and other insect species demonstrate variations in relative quantities of sex pheromone components between individuals (Klun et al., 1980a,b; Roelofs, 1980; Löfstedt et al., 1985; Du et al., 1987; Barrer et al., 1987). The technique that we applied to measure the airborne sex pheromone components of the almond moth is based on passing air over a free-moving single female in a glass cage (Shani and Lacey, 1984). The fact that the calling female can adopt any calling position in any direction might affect both the blend proportion of the airborne pheromone and its trapping efficacy. In addition, it could be that some of the pheromone is retained on the wall surface of the glass cage (Witzgall, 1986) and thus the amount of pheromone collected in the capillary tube is smaller than the quantity actually released. This adsorption onto the glass might also distort the blend composition that is found in the capillary, as compared with the cage composition and the original composition, leading to erratic and erroneous results. The potential discrepancy in blend composition is more crucial to airborne pheromone collection from individual females than the absolute amount collected.

In order to establish the validity of the results obtained with our collection technique, we had to clarify the influence of these two important factors, i.e., gland orientation and possible cage-wall adsorption, on the pheromone blend collected. We therefore monitored the calling position and orientation of the sex pheromone gland of almond moth females in cages mounted on a glass manifold (Shani and Lacey, 1984) and measured both the blend and the absolute amount of the sex pheromone [(*Z*,*E*)-9,12-tetradecadienyl acetate, designated as D and (*Z*)-9-tetradecenyl acetate, designated as M] collected in the capillary tube and washed from the cage surface.

We also studied the pheromone released during the scotophase on three consecutive nights to determine if and to what extent variation in blend composition and quantities occurs with time in individual females (Barrer et al., 1987).

METHODS AND MATERIALS

Collection of airborne pheromone components from single females was conducted in the system developed recently by Shani and Lacey (1984) with a low airflow of 40 ml/min in a cage. Under these conditions, no breakthrough of pheromone was detected within a second pipet mounted immediately downstream from the collection pipet. The technique used in the present investigation was essentially the same as that previously reported, but cotton wool replaced the Poropak Q and glass wool filters in the airstream. Hydrocarbon-free air from a cylinder (General Welding Supply Corp., Westbury, New York) was used in the system, and all capillaries were washed three times with 2–3 μl spectrograde

cyclohexane (Fisher Scientific). The capillaries, which were extensions of the cages (Shani and Lacey, 1984), were made of disposable glass Pasteur pipets (Fisher, 9 in. borosilicate glass 13-678-20D). The cages were washed from the inside with cyclohexane (6–8 × 4–5 μl) into a presealed short capillary (5–6 cm) made of the tapering part of a Pasteur pipet.

All samples were sealed at the top and kept at −5°C until analyzed on a gas chromatograph (GC). Each sample was injected (1 μl) twice, and the difference between the duplicate blend proportion was generally less than 2%. Duplicate measurements for individual females were rejected if they differed by more than 4%. Of the 78 collections (39 for capillaries and 39 for cage washings) that were accepted, duplicate blend proportions varied by 0–1.0% in 55%; 1.1–2.0% in 30%; 2.1–3.0% in 8%; and 3.1–4.0% in 7%. The blend proportion and quantities for the duplicates were assessed by interpolation with standard solutions [D:M 1:1, 2:1, 3:1, 4:1 in the range of 68–340 pg of D and 21–340 pg of M, which were injected intermittently five to six times during a day of GC injections (Shani and Lacey, 1984)]. All GC measurements were performed on a Varian 3500 gas chromatograph, with FID equipped with DB 1701 capillary column, 30 m × 0.25 mm ID, 0.25 μm film, operating in splitless mode, He flow (carrier gas) of 2 ml/min (velocity of 45 cm/sec). The injector temperature was 265°C, and the detector temperature 320°C. The separation at baseline of the two components was achieved by the following programming: Injection at 50°C (hold 1 min), heating to 183°C (50°C/min and hold for 6 min), and then heating to 250°C (50°C/min) and holding for 0.5 min before cooling. Retention time for M was 8.5 min and that for D 8.8 min. The GC was operated at its maximum sensitivity (attenuation 1), and amounts as little as 10 pg of M and D could be detected and measured.

The females in the study were taken from a laboratory culture obtained from Robert Davis, USDA, Savannah, Georgia. All females, being less than 24 hr old, had emerged the morning of the collection. When the pheromone collection extended over three days (see below), the females were kept individually in glass vials in a rearing room when not in the collection cages.

The females in cages were monitored during the collection period (1300–1500 hr which were the first 2 hr of the scotophase), during which time the light intensity was 0.5–1 lux, and temperature was 25 ± 3°C. The calling behavior and position were recorded every 40–45 min, starting at 1300 hr. Thus, four recordings were obtained for each female. Only females that exhibited the same position for at least three recordings were selected for the study of cage washing.

Four calling positions (±20° from the designated direction) were defined: I, female on the cotton wool parallel to and facing into the airflow, with gland extended upwards (Figure 1, a); II, female on the side wall or floor of the cage in a position perpendicular to the airflow (Figure 1, b); III, female on the side

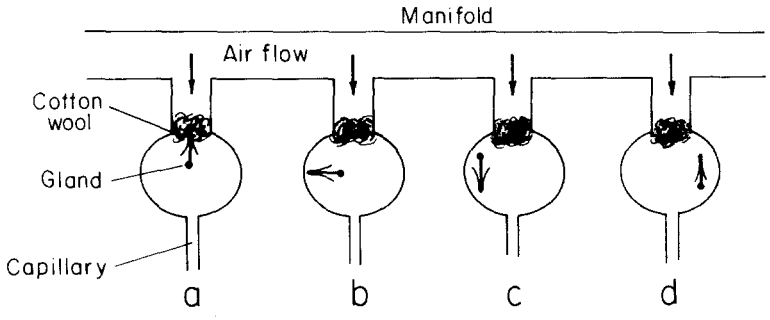

FIG. 1. Calling positions of almond moth females and direction of extended glands in relation to the air flow.

wall or floor of the cage facing the direction of the airflow (Figure 1, c); IV, female on the side wall or floor of the cage in a position facing into the airflow (Figure 1, d). Calling behavior was typified by the gland being extended above the opening of the wings. Noncalling behavior was expressed by the wings being closed together above the back of the moth. In a few cases the gland was hardly visible above the opening between the wings, and noncalling behavior was recorded. Noncalling females rested in the cages in different locations, as did the calling females.

In the three night collections, each female was kept in the cage for 11 hr, and the capillary was replaced every 2–2½ hr by a new one (Shani and Lacey, 1984). The behavior was monitored every hour for the first 5 hr, and thereafter only when the capillary was changed.

RESULTS AND DISCUSSION

The calling behavior of all females monitored during the collection period is summarized in Table 1. The majority of the females (93%) exhibited calling behavior for 2 hr or for part of that time, and we obtained good correlation between behavior and detection of pheromone in the capillaries. In only 1% of females (N = 197) (Table 1) for which noncalling behavior was recorded, was pheromone detected and measured. In 84% of the total females studied (Table 1) pheromone could be detected and measured. In a preliminary study, in which we observed the female behavior continuously for 1 hr, it was found that many females adopt the same position in the cage for a long period (up to 1 hr). In our previous study (Barrer et al., 1987) the females were monitored at 15 to 20-min intervals. Earlier in the current study we found females that stayed in the same position for most of the 2 hr collection period. Thus, four recordings

TABLE 1. CALLING BEHAVIOR OF ALMOND MOTH FEMALES AND PHEROMONE DETECTION IN CAPILLARIES

	Pheromone detected						Total	
	Present		Traces[b]		Absent			
Behavior[a]	*N*	%	*N*	%	*N*	%	*N*	%
Calling	118	60	7	4			125	64
Partial calling	46	23	6	3	6	3	58	29
Noncalling	2	1			12	6	14	7
Total	166	84	13	7	18	9	197	100

[a] Calling—extension of gland was recorded in all 4 monitorings; partial calling—calling behavior was detected in two or three recordings; noncalling—wings closed together and no extension gland was recorded.

[b] Small peaks of one or both components of the pheromone were detected, the peaks being too small to permit accurate measurement.

in this study seemed sufficient, when the same position was kept for a $1\frac{1}{4}$-to 2-hr period.

Although no rigorous study was conducted on the effect of temperature on pheromone release, it was found that on days when the temperature was lower (22–25°C) almost all (90–95%) females called, while fewer females called when the temperature rose to 27–28°C (Webster and Cardé, 1982; Delisle and McNeil, 1987). In previous studies (Coffelt et al., 1978; Coffelt and Vick, 1987) a much smaller percentage (30–46%) of *E. cautella* females called during 1 hr of study or during the entire scotophase. We relate our finding of a very high percentage of calling females to the fact that they were located alone in cages; thus, no effect of other females or of excess of pheromone was perceived by each female. This phenomenon should be taken into account when calling behavior is studied in large groups of females, a situation that is not typical to moths.

The majority of the calling females adopted a position in which the gland was exposed to the airflow, away from the body, as shown in calling positions I and IV (Figure 1, a and d; Table 2). If position II (Figure 1, b), in which the body is also held away from the flow of the pheromone plume, is also taken into account, over 85% of females prefer a position in which no obstacle is mounted in the pheromone path. This percentage is higher than the statistical distribution of three of four positions (χ^2, $P < 0.005$, $df = 1$). The remainder (14%) adopt position III (Figure 1, c), in which the pheromone might be

TABLE 2. CALLING POSITION OF ALMOND MOTH FEMALES IN GLASS CAGES MOUNTED ON COLLECTION SYSTEM (AIRFLOW 40 ml/min)

Calling position[a]	Number of calling females in specific position			
	In total population studied		In sampled population for cage washing	
	N	%	*N*	%
I	60	39	11	28
II	44	28	9	23
III	22	14	10	26
IV	30	19	9	23
Total	156	100	39	100

[a] For details see Figure 1, a–d.

adsorbed on the body of the female, thus reducing the free movement of molecules in the air and diminishing the chance of male attraction.

Position I (Figure 1, a) is the most popular (39%) (Table 2, calling I) of all the positions (and is much higher than 25% of statistical distribution, χ^2, $P < 0.005$, $df = 1$). Sensing headwind probably gives the female moths a sense of direction for the release of pheromone in the most efficient way to attract the males flying upwind. This behavior was also observed in calling *Manduca sexta* females in the field (Richard G. Endris, personal communication).

Sampling of cages to be washed and compared with capillary collection of the airborne pheromone (Table 2) was planned in such a way as to give an equal representation of each calling position. The results of cage washing as compared with capillary collection as a function of calling position are summarized in Table 3. Although swirling air and eddies cannot be excluded, no significant differences were found (F 3, 32; $P > 0.05$, ANOVA) in the blend proportion between the capillaries and the cages in each calling position and among all positions as the total group. It is important to emphasize that the population profile is not distorted between the capillaries and the cages (Figure 2). These findings are crucial for studies of airborne pheromone emitted and collected from individual females. If different proportions had been found in various directions or between cage and capillary, then such measurements would be erroneous and would lead to mistaken conclusions.

The mean quantities washed from the cages are usually larger than, and significantly different from those collected in capillaries (Table 3) ($P < 0.05$, ANOVA). The ratio between the cages and the capillaries is about 1.7 (60–65% of pheromone adsorbed on cage surface) and is similar in all calling posi-

TABLE 3. BLEND COMPOSITION OF ALMOND MOTH SEX PHEROMONE OF CALLING FEMALES FROM CAGES AND CAPILLARIES

Calling position[a]	No. of females	Average proportion (± SE) [D/(D + M)] × 100		Average amount collected (pg) (±SE)		Ratio (pg cage/pg capillaries)
		In capillaries	In cages	In capillaries	In cages	
I	9	61.7:38.3 ± 0.8	63.6:36.4 ± 0.7	528 ± 17	971 ± 44	1.8
II	9	53.2:46.8 ± 1.3	54.5:45.5 ± 1.4	361 ± 23	682 ± 26	1.9
III	9	57.1:42.9 ± 1.1	58.5:41.2 ± 1.2	334 ± 10	524 ± 17	1.6
IV	9	60.4:39.6 ± 1.5	59.9:40.1 ± 1.3	363 ± 15	514 ± 12	1.4
Average		58.1:41.9 ± 1.8	59.2:40.8 ± 1.8	396 ± 50	672 ± 85	1.7

[a] For details see Figure 1, a–d.

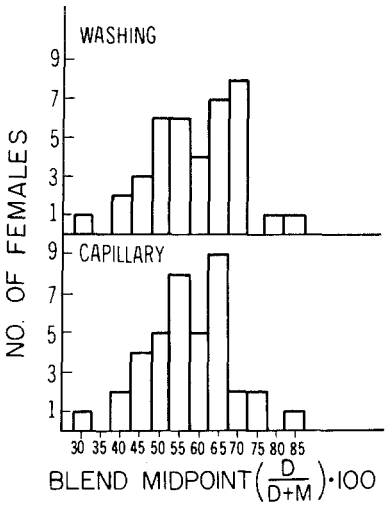

FIG. 2. Population profile of almond moth pheromone blend collection from capillaries and cage washing.

tions ($P > 0.05$, ANOVA). This finding suggests that no (or very little) pheromone is lost or adsorbed on the body of the moth in position III, although adsorption in all positions, because of swirling air and eddies, cannot be excluded. This finding is important for behavioral studies when air is allowed to flow into a system, while in still air most of the pheromone released by the female is adsorbed on the body (Baker et al., 1980; Ramaswamy and Cardé, 1984). Quantities in capillaries of about 100–1400 pg per female and in cages of 220 pg to 3 ng per female, which give a total amount of 300 pg to 3.6 ng per female, are in agreement with previous studies (Barrer et al., 1987, and references within), but lower than the 16.2 ng per female reported recently (Coffelt and Vick, 1987). The blend proportion (D : M) ranged from 41 : 59 to 87 : 13, and no correlation was found between the two variables (blend and quantity).

Five almond moth females were subjected to continuous pheromone collection in cages for 11 hr, starting 3 hr before and continuing up to 8 hr into the scotophase. The collections were performed on three consecutive nights, and the blends and quantities collected in the capillaries are summarized in Figure 3. The females exhibited a calling position for most of the time, even sometimes in cases where pheromone was not found or was found in minute amounts, making it impossible to measure and determine the ratio. Each female emitted a pheromone blend that fluctuated within a relatively narrow range (Barrer et al., 1987). The total amount collected also fluctuated, and, in general, an

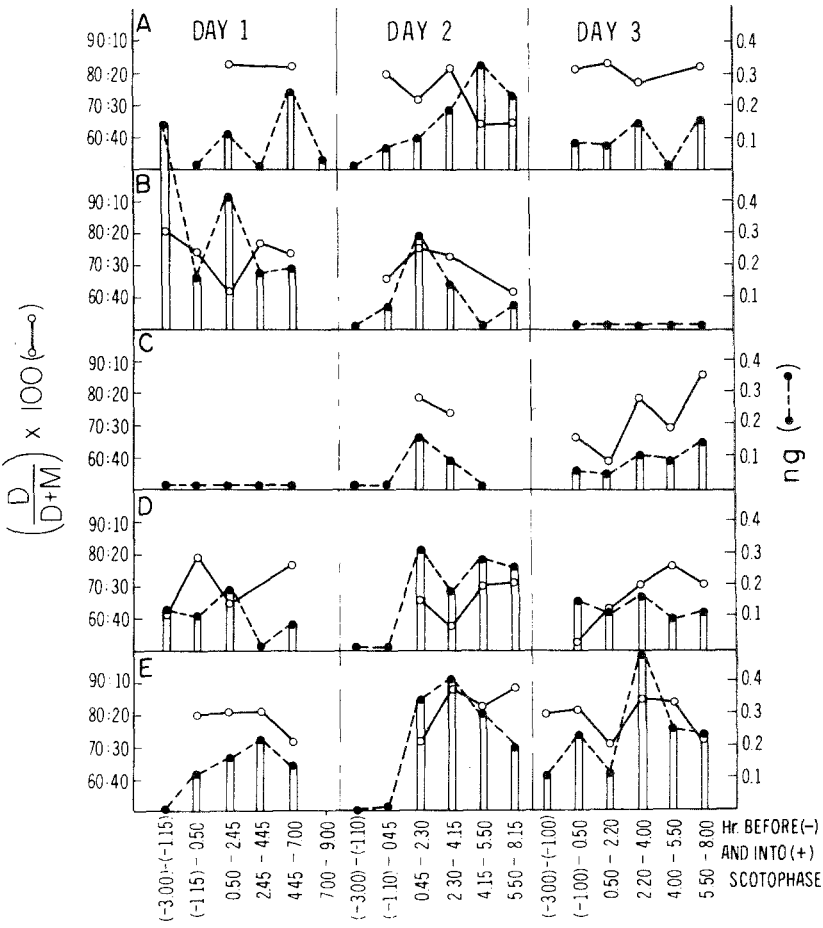

FIG. 3. Pheromone blend and quantity emitted from five individual almond moth females over three consecutive days.

increase in quantity was associated with a shift toward a blend richer in D, the more abundant component in the pheromone blend.

In contrast to our findings, in *Heliothis virescens* the blend composition remained relatively constant throughout the 24-hr period although the absolute amounts did fluctuate (Pope et al., 1982).

The results of our continuous collection experiment support the findings of Coffelt and Vick (1987), who suggested that the pheromone is produced continuously during the scotophase as long as pheromone is released from the gland, albeit preproduction and storage of the pheromone in the gland for the whole

period cannot be excluded. It is not clear whether the change in pheromone blend is controlled during the search for a male tuned to a specific pheromone composition. If this is the case, it means that the female exercises an active emitting control on the release of a specific blend, which can be changed from time to time. These fluctuations in blend and quantity of the pheromone released by single females during the calling period should be taken into account when comparison between results from different research studies are made.

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AIR SAMPLING OF VOLATILE SEX PHEROMONE COMPONENTS IN A CLOSED JAR

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Abstract—A cotton wool plug, used as the source for pheromone release, was placed in closed 1-quart Mason jars, either at the mouth or at the rear of the jar. Air sampling of the two components of the sex pheromone (total 2.2 mg at the source) of the almond moth (*Ephestia cautella*) female showed that the saturation period near the source in still air was 20–22 hr and that far from the source was 40–50 hr, reaching a level of less than 1 ng/ml air. The ratio between the components (*Z,E*)-9,12-tetradecadienyl acetate, designated D, and (*Z*)-9-tetradecenyl acetate designated M, in the air was close to the original ratio for both sampling sites, albeit somewhat richer in the more volatile (*Z*)-9-tetradecenyl acetate (source 77.0:23.0 D:M, air 73.0–74.3:27.0–25.7; source 80.1:19.9, air 77.6:22.4; source 25.1:74.9, air 23.9:76.1 D:M). The total amount of pheromone per milliliter of air was two to three times larger near the source than far from it at the early stages of the evaporation and saturation process. When the amount of pheromone applied to the source was tripled (7 mg), the amount far from the source was almost tripled, or the saturation time was cut by factor of two to three.

Key Words—Air sampling, still air, closed jar, volatiles, pheromones, Lepidoptera, Pyralidae, *Ephestia cautella*, (*Z,E*)-9, 12-tetradecadienyl acetate, (*Z*)-9-tetradecenyl acetate.

INTRODUCTION

Laboratory and field studies of insects behavior under the influence of a mixture of volatiles rely, among other factors, on the quantification of the absolute amounts of the components studied and their blend ratio in the air. The advantage of a laboratory study is that atmospheric factors, such as temperature, wind (speed and direction), pressure, and humidity can be controlled and fixed to our

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needs. Therefore, a careful study of insect behavior in a wind tunnel or in a closed container is much less complicated than a study under field conditions. Although these conditions do not mimic those in nature, we can still get meaningful results. An important factor that should be measured and quantified is the amount of volatiles in the closed system under study. We need to know whether the nature of the source or dispenser from which the chemicals are released will affect the speed of evaporation and the release rate of each component. It is also important to learn how long it takes for the volatiles to reach equilibrium with the source, namely, to "saturate" the atmosphere in the container, for example. This type of information was required for a study in which we observed behavior and mating of the almond moth (*Ephestia cautella*) in a closed jar under "confusion" conditions.

This paper describes a simple technique for air sampling from a closed jar and the rate at which the two components of the sex pheromone of the almond moth permeate the jar from the pheromone source (cotton wool). It also shows that the site of the source in the jar affects the distribution of volatiles within the container. The blend studied consisted of two components, (*Z*,*E*)-9,12-tetradecadienyl acetate [(*Z*,*E*)-9,12-C_{14}OAc designated as D for diene], and (*Z*)-9-tetradecenyl acetate [(*Z*)-9-C_{14}OAc designated as M for monoene)].

METHODS AND MATERIALS

Air Sampling and Preparation of Containers. The type of closed container used in this study was a 1-quart (about 960 ml) Mason jar, whose disposable lid had been replaced with a rubber (2 mm thick) cover beneath which was a layer of aluminum foil. Air was sampled through the cover by means of an interchangable needle of a 30-ml hypodermic gas syringe (Perfektum, Micromate, Poper & Sons, Inc.). Air samples of 20 ($\pm$0.5) ml were withdrawn from the jars with a short needle (5 cm) for mouth sampling and a long needle (15 cm) for rear sampling. The sampled air in the syringe was slowly evacuated (in 2–3 min) into 15–20 μl of spectrograde cyclohexane (C_6H_{12}) (Fisher Scientific), contained in the bottom of a short (4–5 cm) capillary tube. The tube consisted of the narrowing part of Pasteur pipet (Fisher, 9 in. borosilicate glass, 13-678-20D), which had earlier been flamed in the blue zone of a Bunsen burner. The evacuation caused some of the solvent to evaporate, but when the needle was rinsed (3 $\times$ 2–3 μl) into the capillary, the original volume was obtained. After sampling of each jar, the same volume of air that had been withdrawn was introduced into the jar by the syringe. When two samples were taken from the same jar, the air was replaced after the second sampling. After sampling, the syringe was cleaned by pumping fresh air in and out five times. The needle was cleaned from the outside with tissue paper. The capillary tubes were sealed at

the top and kept at −5°C until the pheromone content was determined on a gas chromatograph (GC).

Each jar was equipped, close to the mouth, with a metal hook, on which a copper screen basket (13 × 13 × 10 mm) loaded with cotton wool (30–40 mg) was hung when the pheromone source was placed at the front of the jar. When the source was situated at the rear of the jar, the basket was placed on the glass itself. The two-component mixtures of D and M were prepared from stock solutions in C_6H_{12}. The cotton wool in the basket was loaded with 1 ml of the tested mixture per jar. The solvent was allowed to evaporate from the cotton wool in a hood for 15–20 min, and then each basket was introduced into a jar. The jars were then transferred to the experimental room, where the effect of "confusion" on the behavior of almond moth was studied (12 : 12 light–dark photoperiod, 210 lux, and 28 ± 1°C). The jars were equilibrated for 1 hr before sampling started. Three different solutions were applied as follows:

	Amount (mg/ml)			$\frac{D}{D+M} \times 100$
	D	M	Total	
1.	1.71	0.51	2.22	77.0:23.0
2.	1.71	0.425	2.135	80.1:19.9
3.	1.77	5.27	7.04	25.1:74.9

GC Measurement and Calibration Curves. For each measurement, 1 μl of a C_6H_{12} solution of the tested mixtures was injected into a FID Varian 3500 gas chromatograph, equipped with a DB 1701 capillary column, 30 m × 0.25 mm ID, 0.25 μm film, operating in splitless mode, He (carrier gas) flow 2 ml/min (velocity 45 cm/sec). The separation at baseline of the two components was achieved by the following programming: injection at 50°C (1 min hold), heating to 180°C (50°C/min, and hold for 7 min), and then heating to 250°C. Retention time was 9.2 min for M and 9.6 min for D. Injector and detector temperatures were 280°C and 320°C, respectively.

Calibration curves were based on injections of a single component or of two-component solutions in subnanogram quantities per microliter of solution (Fig. 1a, and b). Standard solutions of D:M in ratios of 1:1, 2:1, 3:1, and 4:1 were prepared in C_6H_{12} and kept at −5°C when not in use. One syringe was used for all solutions since no "cross-contamination" was detected (Shani and Lacey, 1984). The standard solutions showed constancy in response over a period of months, and the ratio between the two components also remained constant. The standard solutions were injected in between the tested samples, which were assessed by interpolation, based on the heights on the peaks. Two or three injections of each sample were made, and the average is shown in the text and with the standard error (±SE) in the tables. The blend proportions in

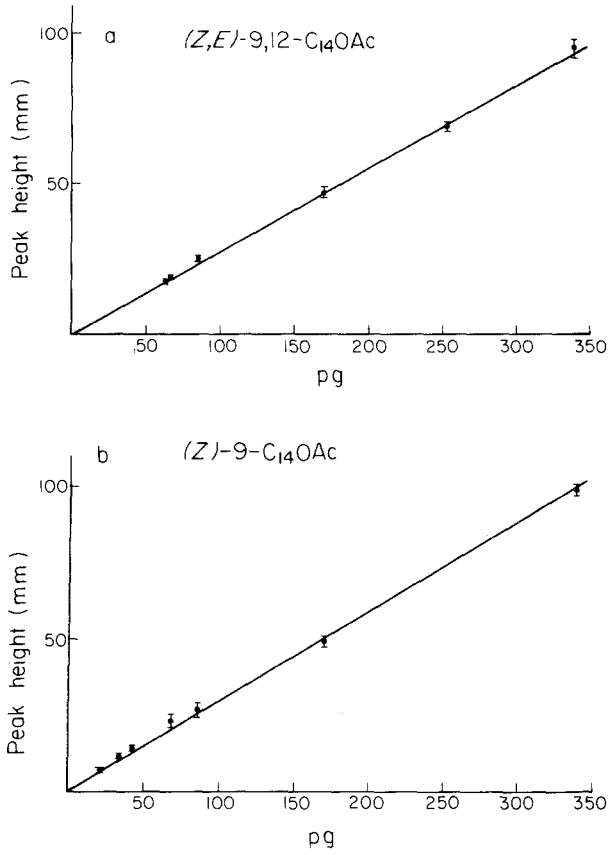

FIG. 1. Calibration curves of (*Z,E*)-9,12-C_{14}OAc (a) and (Z)-9,-C_{14}OAc (b) measured by peak height of GC chromatograms.

each sample varied by 0–1.0% in 42% of all injections; by 1.1–2.0% in 18%; by 2.1–3.0% in 15%; by 3.1-4% in 15%; and by 4.1–5.0% in 10%. All proportions shown are calculated by [(D/(D + M)] × 100.

RESULTS AND DISCUSSION

The first experiment was designed to determine the absolute amounts and the blend ratio of the two components of the test solution remaining on the cotton wool after solvent evaporation (15–20 min). All baskets were loaded with 1 ml of D : M 77 : 23 (2.22 mg) and washed with 10 ml of C_6H_{12}. Samples were assessed both by weighing and by gas chromatography, and the results are

TABLE 1. RECOVERY OF (*Z,E*)-9,12-TETRADECADIENYL ACETATE (D) AND (*Z*)-9-TETRADECENYL ACETATE (M) FROM COTTON WOOL AFTER SOLVENT EVAPORATION

	Amount recovered[a]				
	By weight		By GLC		
Entry	mg	%	mg	%	Proportion [D/(D + M)] × 100
1	2.0	90.1	2.0	90.1	76.5:23.5
2	1.78	80.4	1.85	83.3	75.7:24.3
3	1.79	80.6	1.84	82.9	76.7:23.3

[a] 2.22 mg of mixture applied, proportion 77.0:23.0 (D:M).

summarized in Table 1. In another experiment, the cotton wool was washed after the air sampling (92 hr in jars), and the amounts of blend collected from three samples were 1.9, 1.5, and 1.8 mg. Both these experiments clearly show that the amount lost through evaporation from the cotton wool did not exceed 20% (in one case about 30%) even after 92 hr and, more importantly, that the proportion of the two components at the start of the experiment is very similar to the original composition.

In the subsequent experiments, we studied the effect of a number of factors on the amount of pheromone in the air, the blend composition, and the equilibration time. The pheromone source (2.22 mg except where otherwise stated) was placed either at the mouth or the rear of the jar, and air was sampled from both sides, either near the source or at the other end of the jar. In these experiments, the proportion of the components applied to the cotton wool was changed, as was the absolute amount of blend.

The first experiment in this series was composed of two groups of jars, the first group (jars 1–3) with the pheromone source at the rear of the jar, and the second group (jars 4 and 5) with the source at the mouth of the jar. In both cases, air was sampled at the mouth of the jar. The results for these two sets of jars are summarized in Figure 2 and Table 2. The results show that the evaporation time and equilibrium of the "liquid phase" (cotton wool) and the vapor phase in still air was a slow process, which could take up to 50 hr at a distance from the source, however, the ratio of the components was constant at all times and in all jars ($P > 0.2$, ANOVA), and it differed slightly from the original one. The vapor phase was richer in the more volatile component M (vapor pressure of 1.1×10^{-3} mm Hg at 30°C as compared with 8.3×10^{-4} mm Hg for D) (Hirooka and Suwanai, 1978; see also McDonough and Butler, 1983). Another interesting point is the difference in absolute amount per milliliter of

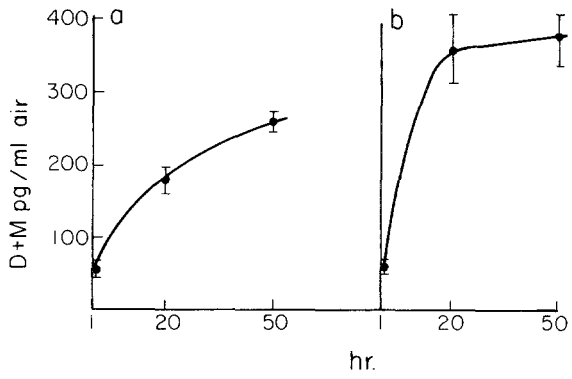

FIG. 2. Rate of air saturation of (*Z*,*E*)-9,12-C_{14}OAc (D) and (*Z*)-9-C_{14}OAc (M) in different closed jars by mouth sampling, with source at the rear (jars 1–3) (a) and at the mouth (jars 4 and 5) (b).

air in the jar between the sampling close to the source (jars 4 and 5) (362 pg after 20 hr) and at a distance from it (jars 1–3) (177 pg after 20 hr). Although equilibrium was reached in the separate jars, it is possible that in still air the equilibrium is so slow that the saturation process in the whole jar does not reach its highest level, even after 50 hr in the latter case.

Therefore, this possible phenomenon was studied by sampling air twice from the same jar when the pheromone source was located at the rear of the jar, as follows. Two separate needles were used for the sampling, one at the mouth and the other at the rear (near the source). The results shown in Figure 3 (average of three jars) demonstrate a large discrepancy in the values for the two sampling sites. The total amounts of the two components after 48 hr in this experiment were 230 ± 21 pg/ml of air at the front, far from the source (which did not differ from 261 ± 16 pg/ml air in jars 1–3) and 673 ± 53 pg/ml air at the rear, near the source (which differed from 377 ± 33 pg/ml air in jars 4 and 5). In both cases the total amounts near the source and away from it were significantly different from one another ($P = 0.01$, ANOVA).

The ratio between the two components was changed in the following experiment (three jars), the value being 80.1 : 19.9 D : M. The source (2.135 mg) and the sampling were at the mouth of the jar. The final vapor phase composition, being richer in the monoene, 77.6 : 22.4 D : M, was somewhat different from the original ratio. The saturation time near the source was 20–22 hr, and the total amount reached was 580 ± 108 pg/ml air (not shown).

In the final experiment, both the ratio and the amount of pheromone applied were changed. A total of 7 mg of mixture, with a proportion of D : M of 25.1 : 74.9, was applied to the cotton wool placed at the rear. Air was sampled

TABLE 2. AIR SAMPLING OF (*Z*,*E*)-9,12-C_{14}OAc (D) AND (*Z*)-9-C_{14}OAc (M) FROM MOUTH OF CLOSED JARS WITH SOURCE AT REAR[a] OR MOUTH[b] OF JAR

			Sampling time									
			After 1 hr			After 20 hr			After 50 hr			
			pg/ml air			pg/ml air			pg/ml air			
Entry	Site of source	Site of sampling	D	M	Proportion[c]	D	M	Proportion[c]	D	M	Proportion[c]	Mean proportion[c]
1	Rear of jar	Mouth	41.5 ± 4.5	13.3 ± 0.8	77.4:22.6	130.2 ± 21.3	46.7 ± 7.1	73.5:26.5	190.7 ± 12.6	70.5 ± 6.2	72.9:27.1	74.3:25.7
2	Mouth of jar	Mouth	43.8 ± 0.3	16.8 ± 1.3	72.3:27.7	262.0 ± 54.0	99.5 ± 20.5	72.5:27.5	279.0 ± 22.0	97.8 ± 14.2	74.0:26.0	73.0:27.0

[a] Average of three jars (1–3) (± SE).
[b] Average of two jars (4 and 5) (± SE).
[c] Proportion calculated as [D/(D + M)] × 100.

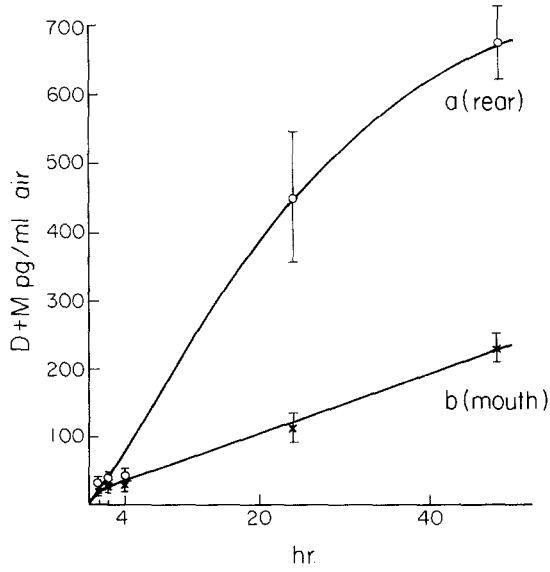

FIG. 3 Rate of air saturation of (*Z,E*)-9,12-C_{14}OAc (D) and (Z)-9-C_{14}OAc (M) in closed jars by rear sampling (near source) (a) and mouth sampling (far from source) (b) while source is at the rear.

at the mouth far from the source. The composition in the vapor phase was found to be 23.9:76.1 D:M. The total amount after 22 hr (saturation time) was 623 $\pm$ 86 pg/ml of air. It seems that the larger amount applied had an effect on the quantity in the air, which was roughly two to three times more than the previous sampling far from the source when 2.2 mg of pheromone was applied. This finding is illustrated (entries 2, 3a, and 5) in Table 3, in which is summarized the set of all experiments including the main different features in each. The maximum amount in the air, in all cases, was less than 1 ng/ml of air, which is less than 1 μg in the whole jar. The vapor pressure of several pheromones was determined and found to be in the range of 0.1 Pa, or about 10^{-6} atm, which is in the range of 10^{-3} mm Hg (Olsson et al., 1983). If we apply the ideal gas equation for our case, we get $n = PV/RT$, $W = MPV/\mathrm{RT}$, which at ambient conditions ($M = 250$, $P \sim 10^{-6}$ atm, $V \sim 0.96$ l; $R = 0.082$, $T = 300°\mathrm{K}$) gives about 10 μg in the whole jar. One reason for this discrepancy might stem from possible adsorption of part of the pheromone on the glass walls of the hypodermic syringe. Another factor could be the slow movement of the pheromone molecules from the interior of the cotton wool sieves to the surface before evaporation into the air. If we view our findings in light of a previous report (Baker et al., 1980) that some evaporated material was adsorbed onto the glass walls, our findings are in good agreement with the calculated value.

TABLE 3. SUMMARY OF AIR SAMPLING EXPERIMENTS

		Original mixture		Found in air			Mean	Site	
Entry	No. of jars	Total applied (mg)	Proportion D:M	Time (hr)	Total pg/ml air	Proportion D:M	Proportion D:M[a]	Source	Sampling
1	3	2.22	77.0:23.0	50	377	74.0:26.0	73.0:27.0	mouth	mouth
2	2	2.22	77.0:23.0	50	261	72.9:27.1	74.3:25.7	rear	mouth
3a	3	2.22	77.0:23.0	48	230	68.8:31.2		rear	mouth
				96	214	68.2:31.8	76.8:23.2	rear	mouth
3b	3	2.22	77.0:23.0	48	673	69.4:30.6		rear	rear
				96	868	70.5:29.5	74.4:25.6	rear	rear
4	3	2.135	80.1:19.9	44	614	77.8:22.2		mouth	mouth
				94	523	78.0:22.0	77.6:22.4	mouth	mouth
5	3	7.04	25.1:74.9	46	599	22.0:78.0		rear	mouth
				94	624	21.7:78.3	23.9:76.1	rear	mouth

[a] Mean proportion over all times and all jars in each experiment.

We found that the blend proportion in air is close to the original proportion in solution, although there is some trend toward a mixture richer in (Z)-9-C_{14}OAc, the more volatile component (higher vapor pressure). From the two experiments with doses of 2 mg and 7 mg, it seems that the amount of pheromone in the air does indeed depend on the amount applied to the cotton wool (Ramaswamy and Cardé, 1984). In the two cases where air was sampled at the same time from near the source and away from it (Table 3, entries 1 and 2, and 3a and 3b), we found different amounts at each site, the larger quantity being measured near the source. This situation is better illustrated by the rate of saturation (Figures 2 and 3, and other data not presented), in which the amount per milliliter of air is always two to three times larger near the source than away from it during the first 20–25 hr of sampling. The fact that concentrations in air depend on the distance from source shows that the saturation process in still air is slow, and the higher concentration is therefore measured near the source.

The study gives a deeper insight into the process of permeation of the pheromone through still air in a container, the permeations depending on the amount of pheromone applied. In order to study the effect of forces of adhesion of the pheromone to cotton wool and glass surfaces on the evaporation, other adsorbing materials such as paper, rubber, glass, and metals should be compared.

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PHYTOHORMONE ECOLOGY
Herbivory by *Thrips tabaci* Induces Greater Ethylene Production in Intact Onions than Mechanical Damage Alone

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Abstract—Herbivory by *Thrips tabaci* affected production of the phytohormone ethylene from living onion foliage. Ethylene analysis was performed by gas chromatography on intact onion tissue. Thrips feeding damage and a crushed thrips extract stimulated significantly greater production of ethylene than could be explained by either one-time or semicontinuous mechanical damage alone, suggesting that ethylene-inducing cues may be transferred to the plant during feeding. This is the first demonstration of increased ethylene production from insect-infested intact plants. This study suggests that herbivores affect both the phytohormone physiology and secondary chemistry of living plants because ethylene has been shown to enhance production of defensive phytochemicals.

Key Words—Onion thrips, *Thrips tabaci*, Thysanoptera, onions, *Allium cepa*, ethylene, herbivory, plant–insect interaction.

INTRODUCTION

The associations between insects and plants are ultimately defined by the chemical interaction of numerous processes and metabolic pathways. Inducible chemical defenses have received much attention (Fraenkel, 1959; Rosenthal and Janzen, 1979; Berenbaum and Feeny, 1981; Fox, 1981; Jermy, 1984; Fowler and MacGarvin, 1986), but the intermediate steps in induction have not been as thoroughly studied. Albersheim et al. (1983) have focused on intermediate steps in the induction of phytochemical defenses by bacteria and fungi. Ryan

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et al. (1986) studied intermediate steps in production of proteinase-inhibitor-inducing factors (PIIF) in relation to insect feeding. Intermediate steps may be dependent on other plant constituents, such as phytohormones, which may exacerbate the problem of developing a comprehensive theory of insect–plant relationships.

The phytohormone ethylene ($CH_2=CH_2$), in the gaseous state, affects the primary chemistry of plants and mediates production of secondary defensive compounds. Its major effects in the primary physiology of plants include induction of fruit ripening (Burg and Thimann, 1959), radial growth of stems (Stewart, 1974), leaf senescence (Jackson and Osborne, 1970), bulb swelling (Levy and Kedar, 1970), leaf epinasty (Crocker et al., 1932), and leaf abscission (Jackson and Osborne, 1970). Secondary phytochemistry is also affected by ethylene produced in response to such stress conditions as mechanical injury (Boller and Kende, 1980; Kimmerer and Kozlowski, 1982), drought (McMichael et al., 1972), flooding (Jackson and Campbell, 1976), chemical damage (Kimmerer and Kozlowski, 1982), and plant disease (Roby et al., 1986). Stress-induced ethylene may result in phytoalexin production, wound healing, and increased disease resistance, which leads to avoidance or tolerance of stress (Yang and Pratt, 1978; Boller, 1983).

Insect damage to plants also leads to increased ethylene evolution from injured tissues. Previous studies measuring ethylene evolution from insect- or arthropod-infested plants (Williamson, 1950; Shain and Hillis, 1972; Powell and Duffey, 1978; Duffey and Powell, 1979; Wien and Roesingh, 1980; Grisham et al., 1987; Kappel et al., 1987; Martin et al., 1988) were conducted on excised plant tissues or organs, with the exception of Williamson (1950), who placed intact mite-infested roses in jars with etiolated seedlings and measured morphological seedling response to ethylene emitted by the infested roses. Ethylene concentration was approximated from ethylene-induced changes in the stem curvature of the seedling.

Because the effects of insect feeding on ethylene production have not yet been rigorously investigated in intact living plants, we conducted studies using onion thrips, *Thrips tabaci* Lindeman, and onions, *Allium cepa* L. Our objective was to obtain evidence that herbivory induces ethylene production in intact plants and to develop techniques that distinguished mechanical induction of ethylene evolution from enhanced production associated with insect feeding.

METHODS AND MATERIALS

Comparison of Herbivory and Mechanical Damage on Ethylene Production by Intact Onion Plants

One-Time Mechanical Damage. Plant cuvettes were designed to allow ethylene collection from intact plants, and to prevent its collection from microor-

ganisms in the soil (Babiker and Pepper, 1984). Glass tubes (23 ml) fitted with rubber septa were placed over the plants, and weather-stripping putty (Mortite) was secured to the bottoms to prevent contact with the soil. The onions were treated in one of two ways: 20 second-instar thrips larvae were introduced or 20 2-mm incisions were made with a sterile 00 insect pin. Initial feeding wounds are produced by puncturing the epidermal layers of plant tissue with the right mandible. The maxillary stylets are inserted, and damage to deeper mesophyll layers is possible. Using a sterile 00 insect pin, both epidermal and mesophyll layers were damaged in a 2-mm incision, resulting in approximately 15.0 mm^3 of damage per day.

Control plants were left undamaged, and empty control tubes were included to check for the presence of ethylene in the collection system. Tubes were stoppered with rubber septa and allowed to incubate for 48 hr. Sample size consisted of 4 plants per treatment. After 24 and 48 hr, ethylene analysis was performed, yielding eight ethylene readings each for uninfested, infested, and mechanically damaged tissues. After the 48-hr reading, plants were weighed to the nearest hundredth of a milligram.

Gas chromatographic analysis followed techniques modified from Babiker and Pepper (1984). After 24 hr, a 1-ml gas sample was withdrawn from the headspace of each vial using a Pressure-lok syringe, series A2 (Dynatech Precision Sampling Corp.) and analyzed for ethylene. A model 5890 Hewlett-Packard gas chromatograph equipped with a flame-ionization detector and a Hewlett-Packard model 3390A integrator recorder were used in the analysis. A 2-m $\times$ 0.318-cm stainless-steel column packed with Porapak N (80–100 mesh) was used, with nitrogen as the carrier gas, operated isothermally at 40°C. A 1004-ppm concentration of ethylene was used as an external standard for quantitation.

Semicontinuous Mechanical Damage. To closely approximate the continuous feeding damage exhibited by thrips, an experiment was conducted in which intact plants were wounded every 12 hr with a sterile 00 insect pin attached to a wire entering the septum. In this manner, damage could be inflicted without opening the tubes. A piece of wire was also inserted into the septa of the remaining tubes. The experimental design was identical to the previous experiment. The sample size consisted of four plants per treatment, and readings were taken after 14, 16, 19, 36, 39, 42, 60, 72, 84, and 94 hr of incubation. After 96 hr, the plants were dried, weighed to the nearest hundredth of a milligram, and the wounds measured to the nearest hundredth of a millimeter.

Thrips Extract. Thrips were placed in a sterile saline solution and macerated into a thick paste. An extract of 10 thrips was placed in each of 10 5-mm wounds made with a sterile 00 insect pin. Mechanically damaged plants were treated in an identical manner to those with the thrips extract, except only sterile saline solution was placed in the wounds. A third of the plants remained unwounded. The sample size consisted of four plants per treatment, and readings were taken every 12 hr for 96 hr. After 96 hr, the plants were dried, weighed

to the nearest hundredth of a milligram, and the wounds measured to the nearest hundredth of a millimeter.

Statistical Analyses

All data were analyzed by one-way analysis of variance using BMDP (Bio-Medical statistical package). Data for the 24- and 48-hr readings were combined for the first experiment, yielding eight readings for each treatment. Differences were considered significant at $P \leq 0.05$ by Duncan's multiple-range test.

RESULTS

A typical gas chromatograph trace comparing infested and uninfested onion tissue is shown in Figure 1. Retention time of ethylene in a 40°C oven was approximately 3.4 min. In the first experiment, thrips-infested tissue produced significantly greater ethylene compared to uninfested plants and to foliage that was mechanically damaged at the beginning of the experiment (Table 1). Ethylene evolution from mechanically damaged, and unwounded plants was not significantly different ($P \leq 0.05$) (Table 1). In experiment 2, thrips feeding wounds and semicontinuous mechanical wounds were not significantly different in size (one-way analysis of variance: $df = 1, 6$; $F = 3.07$; $P \leq 0.05$). Significant differences existed between thrips-infested and mechanically wounded plants and unwounded foliage from 19 to 96 hr of incubation (Table 2, Figure 2). Mechanically wounded and unwounded plants did not produce significantly different levels of ethylene (Table 2, Figure 2). The third experiment produced similar results. Plants inoculated with thrips extract produced significantly greater levels of ethylene than mechanically wounded and unwounded foliage

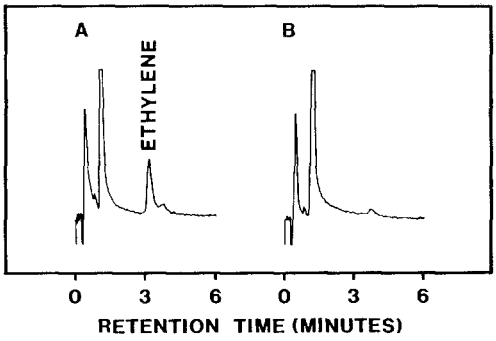

FIG. 1. Gas chromatograph trace of ethylene production from (A) thrips-infested and (B) uninfested excised onion foliage.

TABLE 1. ETHYLENE PRODUCTION FROM INFESTED, UNINFESTED, AND MECHANICALLY WOUNDED INTACT ONION PLANTS

Incubation (hr)	Ethylene production (pmol/g)		
	Infested	Mechanically wounded	Unwounded
24	14.87 ± 3.33[a]	4.59 ± 0.96b	4.50 ± 0.69b
48	21.27 ± 5.10a	5.23 ± 1.00b	3.64 ± 1.23b

[a] Means ± standard errors. Means followed by the same letter in one row are not significantly different (Duncan's multiple-range test, $P \leq 0.05$).

TABLE 2. ETHYLENE PRODUCTION FROM INFESTED, UNINFESTED, AND SEMICONTINUOUSLY MECHANICALLY WOUNDED INTACT ONION PLANTS

Incubation (hr)	Ethylene production (pmol/g)		
	Infested	Mechanically wounded	Unwounded
14	15.94 ± 2.91a[a]	8.11 ± 1.02a	9.02 ± 2.99a
16	12.05 ± 1.99a	10.78 ± 1.46a	5.43 ± 2.50a
19	18.20 ± 4.83a	6.92 ± 1.47b	4.42 ± 1.22b
36	32.29 ± 7.74a	8.03 ± 1.47b	4.79 ± 0.60b
39	41.40 ± 8.83a	12.11 ± 1.66b	6.96 ± 0.81b
42	52.04 ± 7.72a	12.14 ± 1.48b	5.94 ± 1.75b
60	37.38 ± 8.91a	8.41 ± 1.08b	6.02 ± 1.05b
72	70.34 ± 14.38a	16.27 ± 2.62b	4.53 ± 1.26b
84	51.94 ± 11.97a	16.04 ± 2.68b	5.23 ± 1.15b
96	87.40 ± 20.32a	29.35 ± 5.33b	4.39 ± 1.89b

[a] Means ± standard errors. Means followed by the same letter in one row are not significantly different (Duncan's multiple-range test, $P \leq 0.05$).

from 60 to 96 hr of incubation time (Table 3, Figure 3). Ethylene evolution from mechanically damaged and unwounded plants was not significantly different ($P \leq 0.05$) (Table 3, Figure 3).

DISCUSSION

Herbivory by onion thrips stimulated increased ethylene evolution from intact onion plants at levels greater than that produced by mechanical damage. Although actual thrips feeding rates were not measured in this study, herbivory

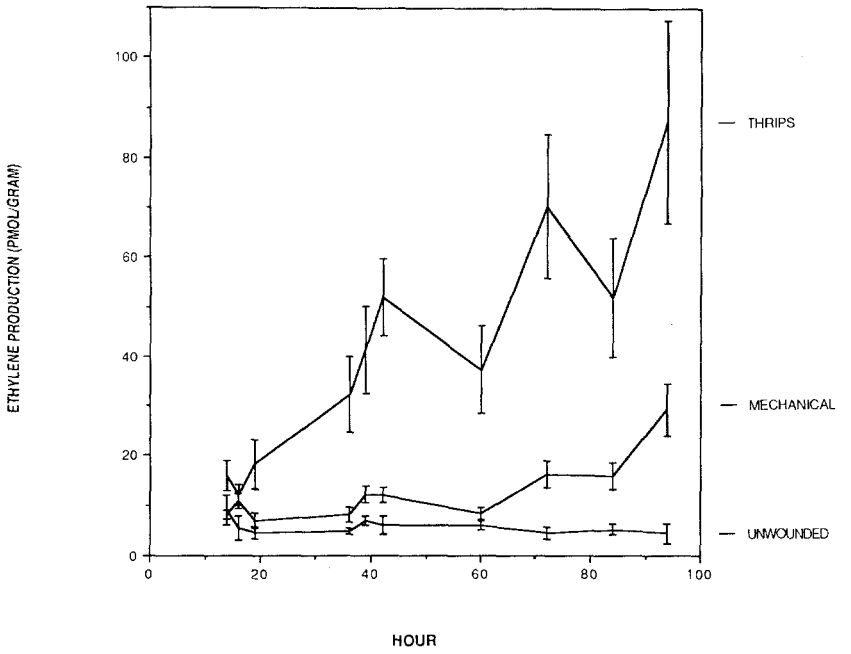

FIG. 2. Ethylene production from infested, semicontinuously mechanically wounded, and unwounded intact onion plants. Vertical lines depict standard errors for the mean.

TABLE 3. ETHYLENE PRODUCTION FROM INTACT PLANTS INOCULATED WITH THRIPS EXTRACT, UNINFESTED PLANTS, AND MECHANICALLY WOUNDED FOLIAGE

Incubation (hr)	Ethylene production (pmol/g)		
	Thrips extract	Mechanically wounded	Unwounded
12	18.06 ± 11.30a[a]	9.26 ± 1.09a	17.79 ± 1.04a
24	18.67 ± 6.90a	8.48 ± 1.01a	21.86 ± 5.57a
36	29.07 ± 7.55a	12.76 ± 1.88a	14.66 ± 2.57a
48	32.28 ± 12.89a	7.36 ± 2.82a	16.23 ± 3.89a
60	52.31 ± 10.85a	7.98 ± 1.88b	11.31 ± 2.83b
72	63.88 ± 22.78a	9.56 ± 3.67b	4.62 ± 1.74b
84	70.37 ± 8.69a	9.56 ± 3.56b	5.96 ± 1.57b
96	90.67 ± 32.36a	10.25 ± 3.63b	14.28 ± 1.99b

[a]Means ± standard errors. Means followed by the same letter in one row are not significantly different (Duncan's multiple range test, $N \leq 0.05$).

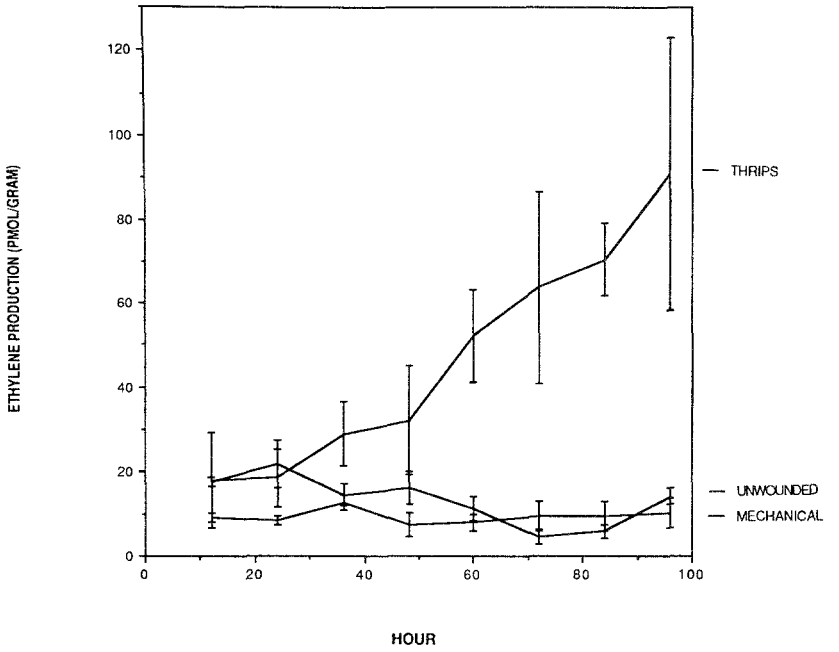

FIG. 3. Ethylene production from plants inoculated with thrips extract, mechanically wounded plants, and unwounded foliage. Vertical lines depict standard errors for the mean.

by *T. tabaci* results in an estimated 4.93 mm^3 of damage per day (Quartey, 1982). This figure is three times lower than the mechanical damage rate of 15 mm^3 per day. Because ethylene production is proportional to the amount of injury (Kimmerer and Kozlowski, 1982), the higher level of mechanical wounding (15 mm^3 per day) should have resulted in increased ethylene evolution. Ethylene evolution from thrips-infested plants was also significantly greater than that produced by plants that were mechanically damaged every 12 hr. These results suggest that the difference was not due to mechanical wounds healing over and producing less ethylene as a consequence. Extracts of macerated thrips also produced significantly greater ethylene evolution than mechanical damage alone. These findings suggest that during feeding, thrips may introduce ethylene-inducing cues. Shain and Hillis (1972) found similar results for ovipositional damage by *Sirex* wasps to *Pinus radiata* trees. Because many insects, including thrips, inject saliva into feeding wounds (Kloft and Ehrhardt, 1959; Miles, 1968a), chemical analysis of salivary glands may provide such evidence. Auxin is an inducer of ethylene biosynthesis (Abeles and Rubinstein, 1964;

Abeles, 1973), and salivary secretions of several insect species contain auxin (IAA) or auxin precursors (Miles, 1968a, b).

Herbivore-induced ethylene evolution may be involved in the intermediate steps of the plant defense system. Ethylene biosynthesis is induced by cell-wall-digesting enzymes (Anderson et al., 1982), plant cell-wall fragments (Tong et al., 1986), and oligosaccharide fragments of invading pathogens (Roby et al., 1986). Plant phenolic levels increase in response to ethylene generated by wounding (Yang and Pratt, 1978) and plant pathogens (Esquerre-Tugaye et al., 1984), indicating its role as a messenger in plant defense mechanisms (Esquerre-Tugaye et al., 1984; Roby et al., 1986). Ethylene initiates production of enzymes, PAL (phenylalanine ammonia-lyase) (Chalutz, 1973) and CAH (cinnamic acid 4-hydroxylase) (Hyodo and Yang, 1971), which are involved in phenolic and phytoalexin formation. Ethylene evolution may be an intermediate step in the production of enzymes that release cell wall oligosaccharide fragments responsible for production of defensive compounds.

Onions possess a complex array of compounds, including several known to have antifeedant and toxic properties, such as phenolics and flavonoids (Fenwick and Hanley, 1985), which increase in concentration following mechanical or insect injury (Edwards and Wratten, 1983). Production of these compounds follows the PAL biosynthetic pathway that is initiated by ethylene (Chalutz, 1973).

Some of the major phenolics present in onion leaves include derivatives of *p*-coumaric acid, ferulic acid, caffeic acid, and protocatechuic acid (Tronchet, 1971a–c). Mechanically injured leaves possess ferulic acid, esculetin, scopoletin, as well as traces of *p*-coumaric acid (Tronchet, 1971c). The antifeedant qualities of these phenolics against insects and other arthropods have been demonstrated by Adams and Bernays (1978) and Valiela et al. (1979).

Onion leaves also possess large quantities of quercitin (Herrmann, 1958), a flavonoid with known antifeedant and toxic properties. Dreyer and Jones (1981) demonstrated the feeding deterrency of quercitin towards the aphid species *Schizaphis graminum* and *Myzus persicae*. The bollworm, *Heliothis zea*, and the beet armyworm, *Spodoptera exigua*, exhibited growth inhibition following ingestion of quercitin and a related compound extracted from quayule, *Parthenium argentatum* (Gray) (Isman and Rodriguez, 1983).

Mechanical injury induces an increase in quercitin in onion plants. Tronchet (1971b) demonstrated that simple finger pressure to onion foliage caused a significant increase in quercitin glycosides. Ethylene is involved in this process through its ability to induce production of flavonoid and phenolic compounds (Yang and Pratt, 1978). Hence, herbivore-stimulated ethylene production plays a significant role in phenolic and flavonoid formation. The lipid-soluble flavonoids may form a matrix that irrevocably binds to protein in a manner similar to tannins, thus explaining the antifeedant properties of quercitin

(Harborne, 1979). Harborne (1979) suggests that a mixture of glycosides of a particular flavonoid would be advantageous in deterring a wide range of herbivores.

Wounded plant cell walls release proteinase-inhibitor-inducing factors (PIIF) responsible for synthesis of proteinase inhibitors (PI) (Ryan et al., 1986). Ingestion of proteinase inhibitors causes increased insect mortality by binding gut proteins (Green and Ryan, 1972; Ryan et al., 1986; Broadway et al., 1986), indicating their effectiveness as insect deterrents. The initial triggering mechanism may be ethylene-inducing substances introduced during insect feeding. These substances may be cell-wall hydrolyzing enzymes, as suggested by Martin et al. (1988). Possible microbial contamination of insect salivary glands may also result in ethylene evolution by the microorganisms themselves or by plant tissues inoculated with the microorganisms (Duffey and Powell, 1979; Grisham et al., 1987; Martin et al., 1988).

Thrips feeding induced significantly greater ethylene evolution in intact plants than would be expected from mechanical damage alone, presumably because thrips introduce cues during feeding that induce ethylene production by the plant. Thrips also may introduce microorganisms that either produce ethylene or induce ethylene evolution from infected plant tissue. Despite the mechanisms involved, plant defense compounds may increase following thrips-induced ethylene production.

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ANCESTRAL SEMIOCHEMICAL ATTRACTION PERSISTS FOR ADJOINING POPULATIONS OF SIBLING *Ips* BARK BEETLES (COLEOPTERA: SCOLYTIDAE)

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Abstract—Three species of *Ips* pine bark beetles in Hopping's group IX (S.L. Wood's *grandicollis* group), *Ips confusus*, *I. lecontei*, and *I. paraconfusus*, are parapatrically distributed in the American Southwest. They share post-Pleistocene altitudinal ecotones with their host pines. Adjacent to these areas of host overlap, we tested the hypothesis that aggregation pheromones produced by male beetles and/or host volatiles are sufficient to elicit the species-specific colonization behaviors typical of these three *Ips* species in nature. A more distantly related species, *I. pini* (Hopping's group IV, S.L. Wood's *pini* group) was used for outgroup comparison. Under the influence of pheromone, males of *I. confusus* and *I. paraconfusus* do not discern among uninfested log bolts of host and nonhost pine prior to bark contact. Males responding to pheromones emanating from infested bolts are similarly undiscriminating. Females of *I. confusus* and *I. lecontei* olfactorily discern the combination of conspecific males in host pine from other possible beetle–pine combinations; females of *I. paraconfusus* do not. Female *I. pini* discerned conspecific pheromone from that of *I. lecontei*. The bark beetle predator, *Enoclerus lecontei*, is attracted by, but does not discriminate among, the male-produced volatiles of these Group IX *Ips* species. These results support a hypothesis that divergence in pheromonal responses by these group IX *Ips* species has evolved following their speciation, having been manifested first in the female sex. Evolutionarily, the derived pheromonal messages have

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preceded their behavioral discrimination by these beetles. Additional species-specific cues may operate between the sexes in the field that may preclude heterospecific pairings.

Key Words—*Ips*, *Enoclerus*, *Temnochila*, Coleoptera, Scolytidae, Cleridae, Trogositidae, aggregation pheromone, cross-attraction, reproductive isolation, speciation, mate recognition.

INTRODUCTION

Adult *Ips* bark beetles (Scolytidae) attack, feed, and oviposit in the phloem and surface xylem of conifers. Host material is usually weakened or freshly dead, and so by its nature ephemeral and patchily distributed. A few "pioneering" males first locate such appropriate but uninfested host material using largely unknown orientation cues. Upon feeding, pioneering males produce frass that contains a volatile aggregation pheromone (Wood et al., 1966). It attracts conspecific males and females, which join the infestation. We refer to these male and female colonists as "joining beetles."

Known *Ips* pheromones are specific enantiomeric blends, usually composed of ipsenol, ipsdienol, and/or *cis*-verbenol, which are derived from host compounds (reviewed in Borden, 1984, 1985; D.L. Wood, 1982). These three terpene alcohols can also serve as allomones that interrupt attraction of sympatric *Ips* species. Thus, whichever species arrives first tends to dominate the host resource (e.g., Birch and Wood, 1975; Birch et al., 1980). As kairomones, these volatiles attract both predators (e.g., Wood et al., 1968; Rice, 1969) and parasites (e.g., Bedard, 1965) of the beetles. Finally, these compounds may attract several scolytid species that share the same host conifer. Cross-attraction may enhance a cohabiting species' chances for finding a suitable host (Birch et al., 1980).

If semiochemical attraction (or interruption) is biologically efficient, then it should be taxonomically informative for scolytid species that cooccur. The genus *Ips* was subdivided taxonomically into 10 species groups by Hopping (1963), and these groups have largely been retained by S.L. Wood (1982). The pheromone of a given *Ips* species is typically unattractive to members of other congeneric species groups (e.g., Lanier and Wood, 1975; Birch and Wood, 1975; Birch et al., 1980). However, olfactory cross-attraction is common among members of the same species group. Broadly sympatric species of *Ips* almost invariably belong to different species groups; conversely, members of the same species group rarely cooccur. Allopatric distributions of closely related *Ips* species may be enforced by the sterile heterospecific pairings that can ensue from their inability to discern each other's premating communications when in sym-

patry (Lanier, 1970a; Lanier and Burkholder, 1974; Lanier and Wood, 1975), a concept originally expounded by Jordon (1905).

Our objectives in this study were twofold: (1) document the degree of olfactory discrimination for pine host and *Ips* beetle semiochemicals that has accompanied the beetles' taxonomic divergence, and (2) assess the extent to which selection in sympatry may have improved such olfactory discrimination. We selected three pairs of *Ips* species representing distinctive increments of taxonomic divergence and which shared some areas of geographic overlap.

The sibling species *I. confusus* (LeConte), *I. lecontei* Swaine, and *I. paraconfusus* Lanier belong to Hopping's group IX (Hopping, 1965). Their grouping is retained as the "*grandicollis*" group by S.L. Wood (1982). In nature, their ranges and common hosts are as follows: *I. confusus* occurs throughout the southwestern United States and northern Baja on pinyon pines; *I. paraconfusus* occurs in California and Oregon on diverse pines (other than pinyon pines); *I. lecontei* occurs in central Arizona and New Mexico southward into central America on diverse pines (other than pinyon pines) (Lanier, 1970a; S.L. Wood, 1982). Their specific rank reflects the general failure of allopatric (Lanier, 1970a) and contiguously distributed (Merrill et al., unpublished) heterospecific populations to hybridize "together with consistent differences in morphological and cytological detail" (Lanier, 1970a). Each species is also electrophoretically distinguishable from the rest by one or more fixed allele differences (Cane et al., unpublished).

Overall similarity of morphometric and electrophoretic characters of *I. confusus* and *I. paraconfusus* suggests their close phylogenetic relationship (Cane et al., unpublished). Lanier and Wood (1975) had earlier established that broadly allopatric populations of *I. confusus* (San Bernardino Mtns.) and *I. paraconfusus* (central Sierra) in California were pheromonally cross-attractive in both laboratory and field experiments when each species produced pheromone in the host pine growing exclusively in its test area. We worked with potentially sympatric populations in the San Bernardino Mountains of southern California, where the pine hosts of the two beetle species intermix in narrow ecotones. Here, we might expect more rapid divergence in ancestrally shared semiochemical communication channels if selection has eliminated genotypes unable to discern between the two closely related species in mixed infestations.

For comparative purposes, we repeated these field assays with a morphologically (S.L. Wood, 1982) and electrophoretically (Cane et al., unpublished) more diverged species trio, *I. confusus*, *I. pini* (Say), and *I. lecontei*. Although *I. lecontei* lacks some of the derived morphological and electrophoretic characters shared by other group IX members, it is nonetheless classified with this group (S.L. Wood, 1982). *Ips pini* belongs to a different species group, group IV or the "pini" group (S.L. Wood, 1982). These three species are reproduc-

tively isolated (Lanier, 1970a; Lanier and Wood, 1975). They cooccur in central Arizona.

METHODS AND MATERIALS

Dates and Locations of Experiments. In the San Bernardino Mountains of southern California (San Bernardino County), *I. paraconfusus* and *I. confusus* cooccur. Their respective hosts, coulter pine, *P. coulteri* D. Don, and single-leaf pinyon, *P. monophylla* Torrey & Fremont, grow sympatrically (Critchfield and Little, 1966) over at least 25 km^2 along the north-central slopes of these mountains (Figure 1). Our experiments with *I. confusus* and *I. paraconfusus* were conducted near this zone of host sympatry during May or July of 1983–1985 (Figure 1).

We tested *I. lecontei* and *I. confusus* in Arizona within 0.5 km of a zone of host sympatry (*P. ponderosa* Douglas and *P. edulis* Engelm., respectively). These pines broadly intermix in the montane canyons of central Arizona, where pinyons occupy the drier slopes. We tested responding *I. lecontei* in July 1986

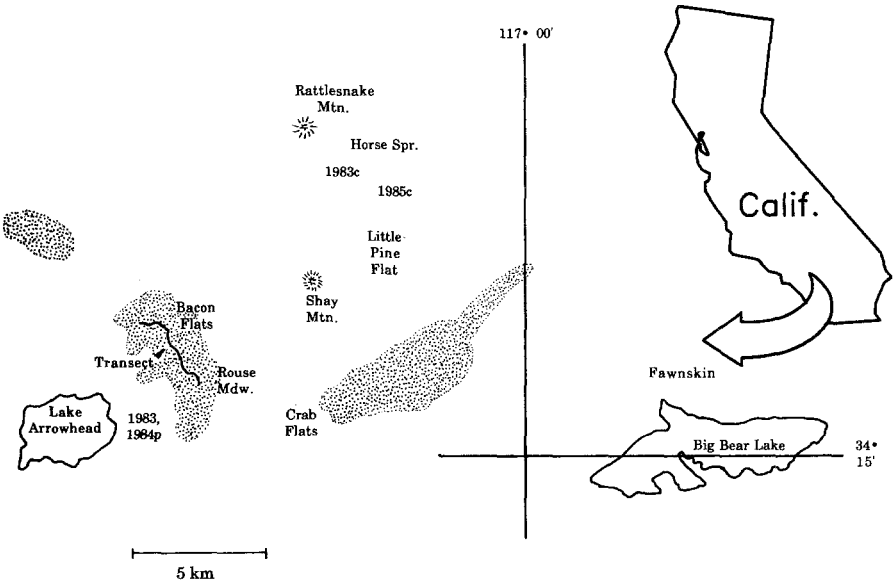

FIG. 1. Map of field sites showing year of experimentation with *Ips confusus* (c) and *Ips paraconfusus* (p) along the north-central slopes of the San Bernardino Mountains in southern California. Site of the transect sample is also marked. Stipled areas are zones of host sympatry: to the north of these areas are pinyon forests; to the immediate south are forests of coulter pine.

in a ponderosa pine commercial thinning above Gaddes Creek in the Prescott National Forest (34°39′N × 112°06′W), 8 km south of Jerome, Arizona (Yavapai County). We also experimented with *I. pini* at this time and location (see below).

Experimental Design. In each experiment we presented semiochemicals produced by two species of *Ips* and their respective host pines. Uninfested pines of equivalent phloem thicknesses were felled, left intact 24–72 hr (±2 hr for a given year) and cut to yield six 15- to 19-cm-diam log sections (bolts) with 1500 cm^2 of bark surface. Outer bark plates were shaved off to facilitate introduction of males. For each of the two pine species in an experiment, four of the six bolts served as uninfested "blank" bolts. They were immediately wrapped with aluminum window screening to prevent volunteer attacks.

The remaining two bolts of each pine species were infested with 25 conspecific males before screening. We term these "bait" bolts. Males were reared from infested material collected within 10 km of host tree sympatry and our experimental sites, except *I. confusus* used in the Arizona tests, which we reared from *P. monophylla* collected in the San Bernardino Mountains. The day preceding experimentation, refrigerated males were individually introduced to a bait bolt through a subcortical nail hole and confined beneath a dimpled screen patch. Those few males (<4%) not producing boring dust after 12 hr were replaced, and the entire bolt was screened. Eight unifested bolts and four bait bolts were used in each experiment. Each bait bolt represented one of the four possible combinations of host–nonhost and conspecific–heterospecific *Ips* infestation.

These 12 bolts were arrayed in four plots 100+ m apart. Each plot consisted of an uninfested host and nonhost bolt 1 m to either side of a central bait bolt. This short distance was chosen as pheromone is necessary to attract male *Ips* into the vicinity of uninfested bolts (Wood and Vité, 1961; Lanier and Wood, 1975) with the exception of *I. latidens* (LeConte) (Miller et al., 1986). Each bolt was surrounded by a 30-cm-diam cylinder of 0.6-cm mesh hardware cloth (ca. 2 cm from the bark surface) lightly coated with heated Stickem Special adhesive (Seabright Enterprises, Emeryville, California). Each bolt was placed atop an upright 1.5-m pipe standard. Entrapped responding beetles were removed daily, rinsed in kerosene, and stored in alcohol for later identification (Bedard and Browne, 1969). To minimize day and position effects, bolts were randomized daily between and within plots such that each plot received a bait bolt and two blank bolts, one of each pine species. Weather, temperature, and wind velocity and direction were noted daily. Experiments ran from four to six days.

In a related experiment, we tested the pheromonal specificity of cooccurring *I. pini* and *I. lecontei* in Prescott National Forest, where both species attack ponderosa pine. Three ponderosa pine bolts were infested, one with 22 conspe-

cific males of either *I. lecontei* or *I. pini*, and one with 11 of each species. A fourth bolt was left uninfested. The males had been taken locally from newly constructed nuptial chambers in logging debris. Bolts were randomized and responding beetles collected on alternate days for six total collections and processed as above.

Beetle Sympatry. We live-trapped *I. paraconfusus* and *I. confusus* in their hosts' ecotone to assess the beetles' sympatry (Figure 1). In May 1984, we set out seven Lindgren funnel traps (Phero Tech Inc., Vancouver, British Columbia, Canada) along a transect. Each trap was baited with a bolt infested with males of both bark beetle species. Two traps were hung in pinyon, four in coulter, and one in jeffrey pine, *P. jeffreyi* Grev. & Balf. Female *Ips* were identified by SEM analysis ($N = 83$).

Identification of Responding Beetles. Beetles were identified by discrete morphological characters where possible. Predatory *Enoclerus* beetles (Cleridae) were identified using keys provided by W.F. Barr (University of Idaho) and determined material in the Essig Museum, University of California, Berkeley. Predatory *Temnochila* beetles (Trogositidae) were identified using Barron's (1971) keys, and confirmed by J.R. Barron (Canadian Department of Agriculture, Ottawa, Canada) and by J.H.C.

All responding *Ips* in our Arizona tests were readily distinguished morphologically. The cryptic sibling species *I. confusus* and *I. paraconfusus* are also easily sexed, but the species lack distinguishing diagnostic macroscopic characters. These two species differ in the density of striations of the female's supracephalic pars stridens stridulatory accessory (Lanier, 1970a). To affirm our ability to discern this difference, and to test our suppositions regarding the beetles' host associations, we measured striation densities of 69 *I. confusus* and 159 *I. paraconfusus* collected from areas neighboring our experimental plots. These females were variously collected from Lindgren funnel traps, naturally infested material, or subsampled from beetles responding in our experiments.

Female beetles were collected into alcohol, later decapitated, and their heads cleaned in xylene and acetone in an ultrasonicator, sputter-coated with 200 Å of gold–palladium, and viewed at 17,000 × magnification using an ISI Super II scanning electronmicroscope. Absolute magnifications were calculated from image diameters of standard latex beads (Ernst and Fullam, Schenectady, New York). Pars stridens striation densities were compared by a *t* test.

Voucher specimens of responding *Ips, Enoclerus*, and *Temnochila* beetle species are deposited with the Essig Museum, University of California, Berkeley; and Auburn University.

Statistical Analyses. Many hypotheses regarding host/mate recognition are addressable by our experimental design. However, as parametric statistics were inappropriate for our data distribution (too many zero values for normalization), we selected three biologically relevant hypotheses for analysis by nonparametric

statistics. We considered each year's experiment in a given location to be biologically independent from any other, and so amenable to independent analyses. We addressed the following hypotheses.

Hypothesis 1—Under the influence of pheromones, male *Ips* coming to uninfested bolts preferentially land at host rather than the sympatric nonhost pine log bolts when discriminatory information is limited to pine volatiles. A Wilcoxon two-sample test was applied to the catches of males numerically ranked across all days at the paired host and nonhost bolts (Sokal and Rohlf, 1981).

Hypothesis 2—Joining males preferentially land at infested bolts of host rather than nonhost pine. Again, a Wilcoxon two-sample test, paired within days, was used. For informal comparative purposes, joining males reponding to the four host-beetle combinations in an experiment were analyzed a posteriori using Friedman's method for randomized blocks (see hypothesis 3).

Hypothesis 3—Joining females preferentially land at one or more of the combinations of host–nonhost bolt infested by con- and/or heterospecific males. Here, both pine volatiles and male pheromones are available. We tested this hypothesis using Friedman's method for randomized blocks (blocked by days), with the four bolt–beetle species combinations as treatment effects (Sokal and Rohlf, 1981). This test was also applied to the catches of *I. lecontei* and *I. pini* in the Arizona tests. For all three hypotheses, statistical rejection was set at $P < 0.05$ for each year's experimentation with each responding beetle species.

In addition, we compared the mean number of *Enoclerus lecontei* (Wolcott) collected daily at the bait and blank bolts for all five experiments. The differences in daily catch between bait and blank bolts were ranked and compared by the pairwise Wilcoxon statistic. We hypothesized that infested bolts would attract proportionally more *E. lecontei* than unifested bolts.

RESULTS

Specific Identities. Our two samples of *Ips* females taken from their two neighboring native California hosts differed significantly in the pars stridens measurement ($P << 0.0005$) (Table 1, Figure 2). Natural host association (coulter vs. pinyon pine) was therefore a reliable distinguishing feature for *I. paraconfusus* and *I. confusus* at this location. The diagnostic differences in pars stridens dimensions that we observed for these two beetle species paralleled those of beetles collected by Lanier (1970a) (both means and variances), who used light microscopy for pars stridens of beetles collected from broadly allopatric populations.

Female *Ips* subsampled from catches in our experiments in the San Bernardino Mountains were, without exception, *I. confusus* at Horse Springs (pin-

TABLE 1. NATURAL PINE HOST, PRESUMED *Ips* BEETLE SPECIES, AND STRIATION DENSITIES OF THE FEMALE PARS STRIDENS[a]

	Pinus monophylla/ Ips confusus	*Pinus coulteri/ Ips paraconfusus*
Number of collection	21	17
Number of beetles	33	29
$\bar{X}$ striation width (μm)	0.650	0.476
Variance	0.0015	0.0005
Range (μm)	0.583–0.711	0.433–0.522

[a] Identities of female Group IX *Ips* collected in the San Bernardino Mountains. These beetles were dissected from naturally infested material. Identification based upon the width of a striation of the supracephalic stridulatory organ of the female.

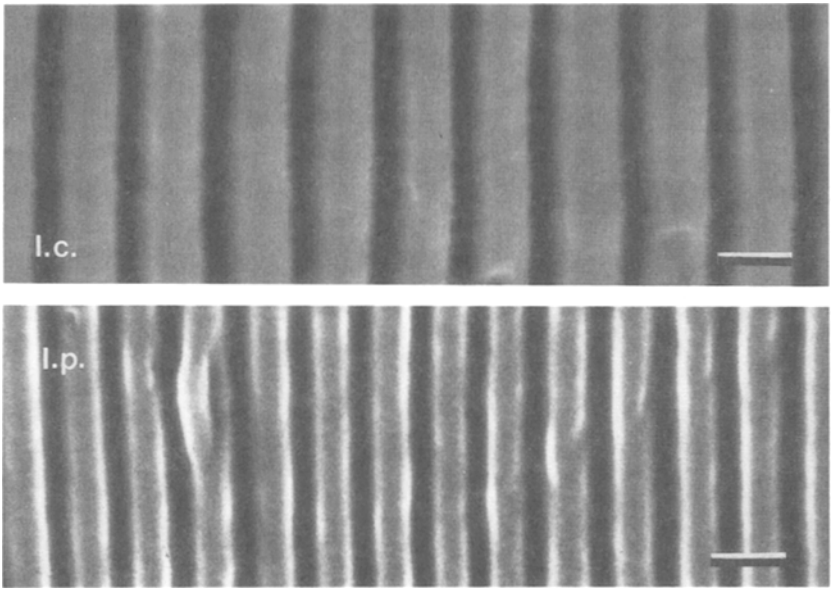

FIG. 2. Surface ridges of the supracephalic pars stridens files of *I. confusus* and *I. paraconfusus*, taken from scanning electron micrographs. Magnifications are identical in the two photographs. Scale line is 1 μm. Mean striation widths: *I. confusus*, 0.632 μm; *I. paraconfusus*, 0.478 μm.

yon site) and *I. paraconfusus* at Lake Arrowhead (coulter site). This was so even though we subsampled treatments most likely to catch the other *Ips* species (e.g., a coulter pine log infested by male *I. paraconfusus* at Horse Springs) (Table 2). We conclude that most (if not all) of the *Ips* beetles responding in our San Bernardino experiments were *I. paraconfusus* at Lake Arrowhead and *I. confusus* at Horse Springs and Little Pine Flat.

Beetle Sympatry. Of the seven Lindgren traps deployed in the zone of host sympatry, four collected only *I. paraconfusus* females ($N = 48$). The remaining three traps, hung in one pinyon and two coulter pines, caught one *I. confusus* each and a total of 32 *I. paraconfusus* females.

Host Acceptance. Males of all *Ips* species readily excavated nuptial chambers. Of 370 males of *I. paraconfusus* and *I. confusus*, 354 excavated nuptial chambers in *P. coulteri* or *P. monophylla*, while nine had died. All 100 males of *I. lecontei* and *I. confusus* in the Arizona experiments started nuptial chambers whether in bolts of *P. ponderosa* or *P. edulis*.

Pioneering Males. Males of *I. confusus*, *I. paraconfusus*, and *I. lecontei* responded indiscriminately to odors of host and nonhost pine bolts in every experiment (Figure 3). Crossover rates (number at nonhost/total catch) ranged from 38% to 62%. Crossover rates of females trapped at blank bolts paralleled those of males in each experiment (±8%). The average daily catch at any one blank bolt was 1.8 males (range 0–29) and 3.2 females (range 0–31).

During the 1983 experiments with *I. paraconfusus* at Lake Arrowhead, all four plots were serendipitously aligned with the prevailing wind direction, such

TABLE 2. STRIATION DENSITIES OF PARS STRIDENS OF FEMALE GROUP IX *Ips* SAMPLED FROM BEETLES TRAPPED IN SEMIOCHEMICAL EXPERIMENTS IN SAN BERNARDINO MOUNTAINS[a]

	Pinyon forest/ *I. paraconfusus* pheromone	Coulter forest/ *I. confusus* pheromone
Number of samples	5	4
Number of beetles	36	43
$\bar{X}$ striation width (μm)	0.615	0.479
Variance	0.0017	0.0006
Range (μm)	0.556–0.706	0.439–0.528

[a] Identities of female Group IX *Ips* collected in the San Bernardino Mountains. These beetles were sampled from our daily trap catches at experimental bolts most likely to attract heterospecifics by olfaction. Identification based upon the width of a striation of the supracephalic stridulatory organ of the female.

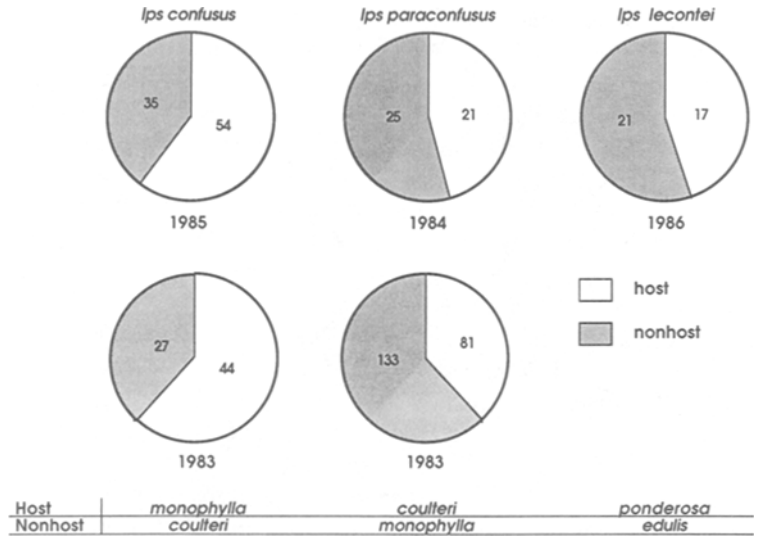

FIG. 3. Olfactory discrimination of uninfested pine bolts by male *Ips* in areas adjoining host sympatry. Responding species named above each pie diagram. Total catches of male *Ips* collected from sticky traps surrounding uninfested bolts of: (1) *P. monophylla* (*I. confusus* host) or *P. coulteri* (*I. paraconfusus* host) in the San Bernardino Mountains; or (2) *P. edulis* (*I. confusus* host) and *P. ponderosa* (*I. lecontei* host) in central Arizona. No significant host discrimination was found for these responding males (see text).

that every blank bolt was either up- or downwind from the infested bait bolt. Pairwise comparisons of the catches at the two blank bolts/plot/day revealed that in 14 of 16 daily comparisons, more beetles were trapped at the blank bolt downwind of the bait bolt than at the upwind blank bolt within a plot (92% of the one-day catches of males and 100% of females of *I. paraconfusus*, 96% of *E. lecontei)*. Overall, downwind blank bolts (which were randomized by pine species) attracted 68% of the males (total $N = 214$), 81% of the females ($N = 231$), and 70% of the responding *E. lecontei* ($N = 290$) trapped at the blank bolts.

Joining Males. In only one experiment were significantly more males caught at the infested host vs. nonhost bait bolts (Figure 4). For a given experiment, 11–76% of joining males were trapped at the nonhost bait bolts. An average of four male *Ips* were caught daily at any one bait bolt (range 0–44 males), or over twice as many males as were attracted to the blank bolts, despite the close proximity of the bolts within a given plot. No consistent numerical preferences for conspecific pheromones were apparent for males joining infested bolts (Figure 5). In applying the same Friedman's test to joining male catches

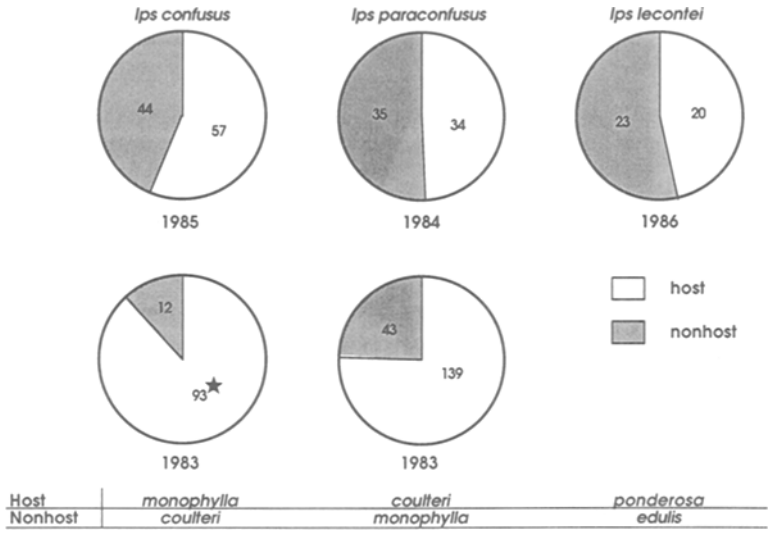

FIG. 4. Olfactory host choices by sympatric male *Ips* trapped at infested bolts in areas adjoining host sympatry. Total catches of male *Ips* collected from sticky traps surrounding infested bolts of (1) *P. monophylla* (*I. confusus* host) or *P. coulteri* (*I. paraconfusus* host) in the San Bernardino Mountains; or (2) *P. edulis* (*I. confusus* host) and *P. ponderosa* (*I. lecontei* host) in central Arizona. Among joining males, only *I. confusus* in 1983 (starred) significantly preferred host to nonhost.

as for the joining female data, the only significant combinations revealed were: (1) a preference by male *I. confusus* in 1983 for the host–conspecific combination and (2) a lesser catch of male *I. confusus* at the *confusus–coulter* combination in 1985.

Joining Females. Females of *I. confusus* arrived in significantly greater numbers at host (pinyon) bolts infested by conspecific males than at the other combinations with coulter pine and/or *I. paraconfusus*. Similarly, female *I. lecontei* arrived in significantly greater numbers at host (ponderosa) bolts infested by conspecific males than at the other combinations with pinyon pine and/or *I. confusus* males. In contrast, female *I. paraconfusus* did not significantly prefer a given host–beetle combination in experiments with *I. confusus* and pinyon pine (Figure 6). Disproportionately more female than male *Ips* were captured at bait than blank bolts in every experiment (Table 3).

Predators. The clerid beetle *E. lecontei* was the most abundant predator of adult *Ips* trapped during our experiments. Within each experiment, significantly more *E. lecontei* were trapped at infested bait bolts than at blank bolts [$\bar{X}$ = 1.6–11.3/infested bait bolt/day (N = 541 *E. lecontei* total) vs. $\bar{X}$ = 1.5–

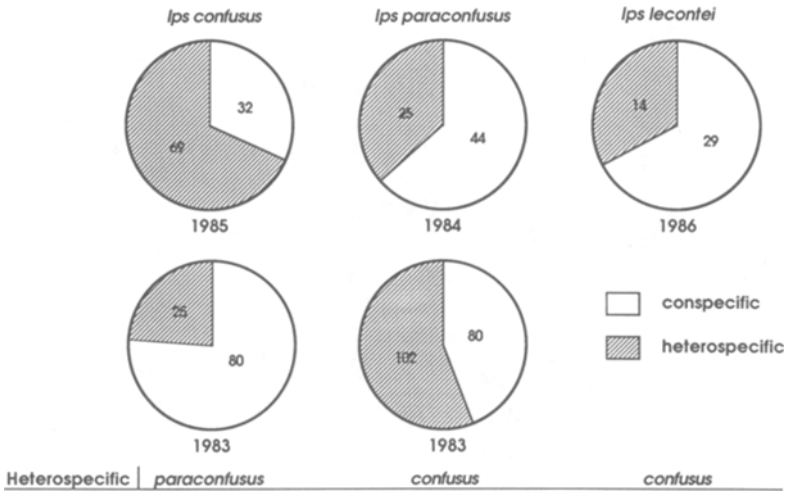

FIG. 5. Olfactory choices for infesting beetle species by male *Ips* in areas adjoining host sympatry. Choices are between conspecific and heterospecific males of the local group IX *Ips* (data as in Figure 6).

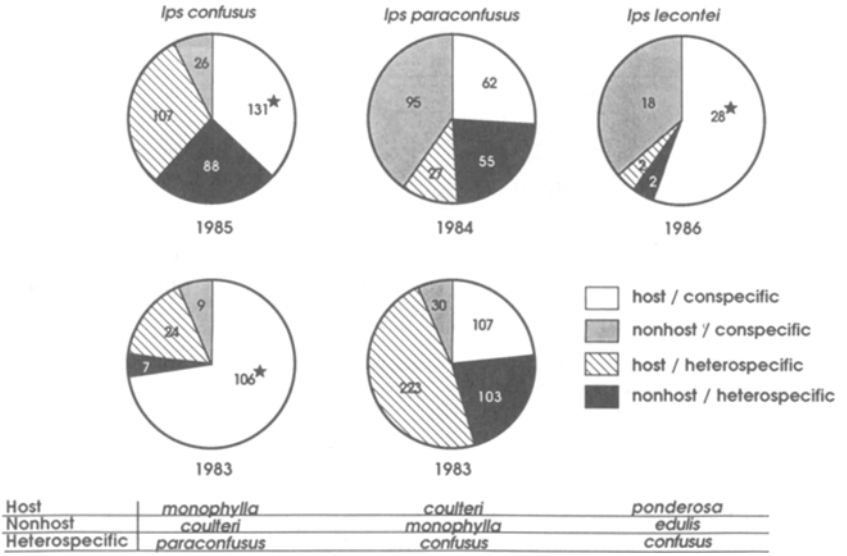

FIG. 6. Olfactory host and male choices by female *Ips* attracted to infested bolts in areas adjoining host sympatry. Total catches of female *Ips* collected from sticky traps surrounding bolts infested with conspecific or heterospecific males in (1) *P. monophylla* (*I. confusus* host) or *P. coulteri* (*I. paraconfusus* host), sympatric in the San Bernardino Mountains; or (2) *P. edulis* (*I. confusus* host) and *P. ponderosa* (*I. lecontei* host), sympatric in central Arizona. A star marks the pine-beetle combination in a particular experiment that attracted significantly more female *Ips* beetles than the other three possible pine-beetle combinations tested.

TABLE 3. *Ips* OPERATIONAL SEX RATIOS (*N* FEMALES/*N* MALES) FROM *Ips* BEETLES TRAPPED IN SEMIOCHEMICAL EXPERIMENTS[a]

	Ips confusus		*Ips paraconfusus*		*Ips lecontei*	
Year	Blank	Bait	Blank	Bait	Blank	Bait
1983	2.50	3.45	2.44	3.45		
1984			2.50	3.57		
1985	0.97	1.39				
1986					0.68	1.16

[a]Operational sex ratios of *Ips* species responding to uninfested versus infested bolts in our experiments, expressed as the ratio of females to males. Larger numbers indicate proportionally more females. Note that the infested bolts consistently attract proportionally more females than the uninfested bolts (see text).

9.0 *E. lecontei*/blank bolt/day, (N = 635 total)]. We collected only four *E. moestus* (Klug), all in California. The only responding predatory trogositids were 28 *Temnochila chlorodia* (Mannerheim).

Olfactory Discrimination between Species Groups. We found marked semiochemical specificity between sympatric *I. pini* and *I. lecontei* (Table 4). Significantly more male and female *I. lecontei* were caught at bolts infested by conspecific males than at any other bolts. On two of six days, no *I. pini* were caught. For the remaining days, all but one *I. pini* was caught at the bolt infested with conspecific males. No *Ips* were caught at the uninfested bolt in this experiment. Our experimental design did not permit assessment of mutual inhibition, as the mixed infestation had only half as many males of each of the two *Ips*

TABLE 4. IDENTITIES OF *Ips* AND *Enoclerus* RESPONDING TO PHEROMONES PRODUCED IN PONDEROSA PINE IN ARIZONA EXPERIMENT (JULY 1987)[a]

Responding beetle species	22 *Ips lecontei*	22 *Ips pini*	11 each species	Uninfested bolt
Ips lecontei				
Males	24	2	8	0
Females	35	0	5	0
Ips pini				
Males	0	11	1	0
Females	0	11	2	0
Enoclerus lecontei	36	42	17	5

[a]Cumulative catches of sympatric *I. lecontei*, *I. pini*, and *E. lecontei* at bolts of their common host, *P. ponderosa*, infested with *I. lecontei* and *I. pini* in central Arizona.

species as did the purely infested bolts. Significantly more *E. lecontei* came to the infested bolts relative to the uninfested bolt.

DISCUSSION

We draw four summary conclusions from these experiments with select species of southwestern *Ips* bark beetles: (1) Responding males of *I. confusus*, *I. lecontei*, and *I. paraconfusus* do not distinguish between olfactory cues emanating from uninfested host and local nonhost pines. (2) Joining males of these three group IX species are pheromonally cross-attractive, whereas males of cooccurring members of different species groups (*I. lecontei* vs. *I. pini*) distinguish their conspecific pheromone from that of the other species group. (3) Females of two of the three species of *Ips* tested are more attracted to the combination of odors from conspecific males infesting the local host pine than other combinations of cooccurring host–beetle species. (4) The bark beetle predator, *E. lecontei*, responds to male-produced volatiles of group IX *Ips* indiscriminately.

These results with adjoining *Ips* populations, taken together with paleobotanical evidence and earlier *Ips* pheromonal research in allopatry, are consistent with the hypothesis that specificity of semiochemical communication among species of *Ips* has followed rather than accompanied their speciation. The bark beetle predator, *E. lecontei*, accommodates these evolutionary changes in pheromones by remaining a kairomone generalist. The degree of *Ips* pheromonal divergence and discrimination has apparently been little modified by host association, predation or parasitism pressures, or prolonged contact with populations of closely related beetle species. Rather, their pheromonal cross-attraction directly reflects their taxonomic relatedness (Table 5). The dualistic role of *Ips* pheromones for signaling the presence of suitable host substrates as well as species identity may explain the asymmetry between female and male *Ips* in their relative abilities to discriminate conspecific from heterospecific pheromones.

Semiochemical Generality of Males. Males from sympatric populations of *I. paraconfusus*, *I. confusus*, and *I. lecontei* did not distinguish olfactory cues emanating from either (1) uninfested hosts when challenged with nonhost pine or (2) bolts infested with conspecific males when challenged with sibling heterospecifics. These male beetles and their *Enoclerus* predators seemed to distinguish individual uninfested bolts from infested bolts within our plots. Our evidence includes consistent sex ratio differences of responding *Ips* (Table 3) and greater numbers of *Ips* and *Enoclerus* beetles caught at the infested bolts relative to the adjacent uninfested bolts. The sex ratio differences may reflect either greater female responsiveness to pheromone (Wood et al., 1966) or a pheromonally mediated male spacing phenomenon (Byers, 1983). The involve-

TABLE 5. REPORTED PHEROMONAL FIELD RESPONSE OF *Ips* PINE BARK BEETLES CHALLENGED WITH BEETLE-PRODUCED HETEROSPECIFIC PHEROMONE[a]

Taxonomic relationship	Full cross-attraction	Partial cross-attraction	No cross-attraction
Same *Ips* species group	hopp : conf[8] gran : conf[7] para : conf[4,9] hopp : gran[7] mont : para[4]	conf : para[4,9] leco : conf[9] conf : mont[4] mexi : conc[4] inte : plas[2] plas : inte[2] mont : conf[4]	
Sum	5	7	
Different *Ips* species group	avul : call[6]		leco : pini[9] pini : leco[9] pini : para[3,5] para : pini[3,5] gran : call[1,6] avul : gran[6] gran : avul[1,6] call : avul[1,6]
Sum	1	0	8

[a] Degree of pheromonal specificity for *Ips* species when pairs of species were compared in field olfaction studies. Studies challenged *Ips* beetles of one species ("a") to discriminate pheromone emanating from bolts infested by conspecific ("a") and heterospecific ("b") males during field olfaction bioassays. Species pairs listed as "a : b." Species abbreviations are: avul (*I. avulsus*), call (*I. calligraphus*), conc (*I. concinnus*), conf (*I. confusus*), gran (*I. grandicollis*), hopp (*I. hoppingi*), inte (*I. integer*), leco (*I. lecontei*), mexi (*I. mexicanus*), mont (*I. montanus*), para (*I. paraconfusus*), pini (*I. pini*), and plas (*I. plastographus*). Summarized from data in Vité et al. (1964)[1], Lanier (1970b)[2], Lanier et al. (1972)[3], Lanier and Wood (1975)[4], Birch and Wood (1975)[5], Birch et al. (1980)[6], Lewis and Cane (unpublished)[7], Cane and Wood (submitted)[8], and the present manuscript[9].

ment of olfactory anemotaxis is suggested by our larger catches of *Enoclerus* and male *Ips* at uninfested bolts downwind of the central infested bolt (relative to the upwind blank bolt) during our 1983 *I. paraconfusus* experiments. Olfactory anemotaxis by *Dendroctonus brevicomis* to pheromone has been demonstrated in the field (Tilden et al., 1979; Byers, 1988).

Males of sympatric populations of group IX *Ips* species perform no better in semiochemical discrimination than allopatric species pairs of this species group. Supporting evidence has been reported for various pairs among *I. confusus*, *I. hoppingi*, *I. grandicollis* (Eichhoff), *I. montanus* (Eichhoff), and *I. paraconfusus* (Table 5). Males of these species pairs are pheromonally cross-attractive. Conversely, males of sympatric *Ips* species pairs each from a differ-

ent species group, such as Hopping's groups IV, IX, and X, show little or no cross-attraction for each other's pheromones (Table 5). We find no evidence that significant divergences in the ancestral olfactory host and species-specific recognition systems have evolved for the males of group IX *Ips* species, despite their contiguous geographic distributions, mutual reproductive isolation, and the apparent adaptive merits of such specificity for minimizing search time, exposure to predators, and successful mate attraction in sympatry. Semiochemical specificity among these species of *Ips* beetles is explained better by taxonomic relationship than their degree of ecological cooccurrence.

Males of all three species of group IX *Ips* used in our experiments readily excavate nuptial chambers and produce cross-attractive pheromones in nonhost pines. However, in our samples of females (the morphologically discernible sex) from naturally infested pines bordering the zone of sympatry in the San Bernardino Mountains, we found no evidence of *I. paraconfusus* or *I. confusus* infesting nonhost pines. Unfortunately, we failed to find pines infested by either of these beetle species in the regions of host overlap. Our limited trapping data in the area of host sympatry suggests that both beetle species do indeed occur together. We offer two proximal explanations for host association: (1) Males infest pines randomly, but responding females only join conspecific males that have infested a host pine. (2) Pioneering males not under the influence of pheromone are host-specific. Our methods do not discern among these hypotheses. The first hypothesis seems less tenable, as we have experimental field evidence that females readily join conspecific males in nonhost pine logs (Fox, Wood and Cane, unpublished). It should be noted that Lanier (1970a) reported a few instances in which group IX *Ips* species naturally colonized nonhost pine species, sometimes resulting in a given gallery system being shared with heterospecific beetles.

Semiochemical Specificity of Females. Unlike males, females of *I. confusus* and *I. lecontei* preferred conspecific males infesting host pine bolts over all other olfactory host–beetle combinations (Figure 6). Female selectivity suggests the existence of species-specific differences between the pheromones of sympatric group IX beetle populations as well as between the volatiles of the pines. Thus, the discriminatory response can be sex-specific among group IX *Ips*. Pheromonal discrimination also can be an asymmetrically possessed attribute of some continguously distributed species pairs. In the San Bernardino Mountains, female *I. paraconfusus* failed to respond preferentially to conspecific males in their host logs, which contrasts with the discriminating sympatric females of its sibling species, *I. confusus*. This finding has precedent. Sexual asymmetries in ethological barriers to hybridization are known for pairs of species of *Drosophila* flies (Kaneshiro, 1983) and *Xiphophorus* fish (Ryan and Wagner, 1987), among other examples. In these cases, one species' females

respond only to conspecific courtship, whereas females of the second species respond to courtship by males of either species.

Complete pheromonal discrimination by both sexes of a pair of sympatrically distributed species of *Ips* is only attained between species of greater phylogenetic divergence, such as *I. lecontei* and *I. pini* (Table 4). Lesser catches of either of these two *Ips* species at a bolt of mixed infestation suggests some mutual inhibition of attraction. Beetle pheromones, rather than host volatiles, are clearly responsible for attraction to male-infested bolts, as evidenced by the rarity of *Ips* individuals caught at the uninfested bolt. Our result may illustrate the mutual inhibition of pheromonal attraction, as reported between *I. paraconfusus* and *I. pini* (Birch and Wood, 1975).

Predators. More adults of the bark beetle predator, *E. lecontei*, were trapped at the bolts infested with male *Ips* than the adjacent uninfested bolts. Adult *E. lecontei* did not discriminate among any sympatric pairs of *Ips* species. This result is not surprising, as this beetle is widely associated with *Ips* species of the American West, upon which it feeds (Berryman, 1966). Our results corroborate earlier studies (e.g., Wood et al., 1968) in showing that the volatiles emanating from *Ips*-infested logs attract *E. lecontei* as well as *Ips*.

Antiquity of Beetle Sympatry. The incomplete olfactory discrimination we see today among these sympatric populations of sibling *Ips* species may simply reflect the recency of their distributional contact. Our samples of live beetles in pheromone-baited Lindgren traps indicate that both species do sometimes fly together in these areas of host sympatry. However, the narrow altitudinal ecotones of the beetles' host pines are clearly thousands of years old. Macrofossil plant assemblages from Holocene *Neotoma* (packrat) middens demonstrate the 5000- to 12,000-yr-old antiquity of the shared altitudinal ecotones of some of these pines in the American Southwest, and indirectly evidence the sympatry of their associated *Ips* beetles [*Pinus ponderosa* and *P. edulis* (Betancourt and Van Devender, 1981; Cole, 1982; Betancourt, 1984); *P. ponderosa*, *P. discolor*, and *P. edulis/monophylla* (Thompson and Van Devender, 1982); *P. monophylla* and *P. ponderosa* (Wells and Berger, 1967); also Pliocene oak–conifer pollen assemblages that "approximate today's foothill and slope vegetation of central and southeastern Arizona" (Gray, 1960)]. We unfortunately have no fossil *Ips* for the relevant time period with which to confirm the historical distribution of these beetles.

If contemporary host associations of these group IX *Ips* beetles have not resulted from recent host switches, then the post-Pleistocene ecotones of their host pines likely also have been zones of sympatry for the beetles for over 10,000 years, or about 30,000 beetle generations [2–4 generations/year (Schultz and Bedard, 1987)]. During this time period, the generality of the olfactory response of both the clerid predators and males of *I. confusus*, *I. paraconfusus*,

and *I. lecontei* have continued apparently unchanged from the ancestral state. In contrast, mate–host discrimination of females of *I. confusus* and *I. lecontei* has evolved toward species specificity.

Dualistic Role of Ips *Pheromones.* Why has selection seemingly overlooked the males' olfactory discrimination between conspecifics and sympatric sibling *Ips* species, when females show significant preferences for what must be diverged semiochemical cues? Similarly, in the northern Rocky Mountains, where western and eastern races of *I. pini* presumably introgress, female *I. pini* better discriminate the proportional differences in enantiomeric composition of pheromones of the eastern and western races than do the males (Lanier et al., 1972). We believe the explanation for such asymmetric discrimination lies with the dualistic nature of the *Ips* pheromone system, which contrasts with that of many other insect species, especially moths, whose pheromones serve solely in mate attraction and discrimination (Greenfield and Karadinos, 1979; Cardé and Baker, 1984; Löfstedt and van der Pers, 1985). For bark beetles (D.L. Wood, 1982; Borden 1984, 1985) and pine weevils (Phillips and Lanier, 1986), the pheromone serves to both attract potential mates and advertise suitable food substrates.

Pioneering male *Ips* confront tremendous odds against locating suitable hosts. Consequently, most males rely upon signals from conspecific males for discovering hosts. Successful establishment is perceived over a considerable distance by searching males using the same pheromonal system that attracts females (summarized in D.L. Wood, 1982). Species-specific differences in pheromonal attributes of closely related *Ips* species are often of a subtle quantitative nature, such as enantiomeric ratios, which could be obscured at a distance from the source. In not responding to such distant and imperfect, although conspecific, pheromonal messages, overly discerning males may miss suitable host material and risk forfeiture of reproduction altogether. For males, the pheromone serves mainly to localize a conifer in suitable condition for attack.

In contrast, the pheromone primarily serves females in identifying the species of infesting male. Less discerning females risk a heterospecific pairing leading to an inviable clutch of eggs (Lanier, 1970a; Merrill et al., unpublished). Among reproductively isolated sibling species for which a pheromone serves both to advertise ephemeral, scattered hosts and to attract mates, such as those of *Ips*, we predict that prolonged contact will produce a sexual asymmetry in pheromonal discrimination. The colonizing sex continues to be a pheromone-generalist (here, *Ips* males), but the sex responsible for mate recognition will evolve greater specificity in its pheromonal discrimination. As evolutionary divergence proceeds, absolute pheromonal discrimination between the species eventually ensues for both sexes (as illustrated by *I. pini* and *I. lecontei*, members of groups IV and IX, respectively), permitting broad geographical overlap.

Finally, mutual inhibition of pheromonal attraction may evolve (Birch and Wood, 1975).

Intraspecific male pheromonal competion, vis-à-vis sexual selection, may catalyze the initial divergences in bark beetle pheromone constitution (West-Eberhard, 1984). It requires heritable pheromonal variation among individual conspecific males within populations and heritable behavioral preferences among females for this variation. As noted by West-Eberhard (1984), male *Ips* undertake considerable parental investment risks in establishing subcortical nuptial chambers. We might therefore expect greater sexual selection among females competing for access to males. The taxonomically diagnostic differences in pars stridens morphology between female *Ips* (Barr, 1969) may reflect such an evolutionary response in the female's courtship stridulation.

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AGGREGATION PHEROMONE OF DRIEDFRUIT BEETLE, *Carpophilus hemipterus* Wind-Tunnel Bioassay and Identification of Two Novel Tetraene Hydrocarbons

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Abstract—A male-produced aggregation pheromone was demonstrated in *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae) using a wind-tunnel bioassay. Both sexes responded to the pheromone, but the beetles flew in the wind tunnel only after they had been starved for at least several hours. The attractiveness of the pheromone was greatly enhanced by volatiles from a food source, and combinations of pheromone and food volatiles typically attracted 3–10 times more beetles than either source by itself. A variety of food-related sources of volatiles were effective. These included apple juice; a mixture of baker's yeast plus banana; the pinto bean diet used for rearing this beetle; the chemicals propyl acetate, ethanol; and a mixture of acetaldehyde, ethyl acetate, and ethanol. The pheromonal activity resided with a series of 10 male-specific, unsaturated hydrocarbons of 13, 14, and 15 carbon atoms. These were partially separated by HPLC. No single compound was absolutely required for pheromonal activity to be observed, and various subsets of these compounds were active. The most abundant component was (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene. One minor component was (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene. These structures were proven by synthesis. Together, the synthetic compounds were as active in the wind tunnel as the beetle-derived pheromone.

Key Words—Pheromone, aggregation, synergism, hydrocarbon, driedfruit beetle, *Carpophilus hemipterus*, Coleoptera, Nitidulidae, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene.

INTRODUCTION

Carpophilus hemipterus (L.) (Coleoptera: Nitidulidae) is a cosmopolitan pest that attacks a large number of agricultural commodities both before and after harvest (Hinton, 1945). It also is able to vector microorganisms responsible for the souring of figs (Hinton, 1945), and mycotoxin-producing fungi that contaminate corn (Wicklow, 1989).

Field traps have been used to monitor or control this and other nitidulid species, and much research has gone into the development of trap baits. Smilanick et al. (1978) determined that a 1:1:1 mixture of acetaldehyde, ethyl acetate, and ethanol is an effective bait for *C. hemipterus*. Alm et al. (1985, 1986) demonstrated that esters such as propyl propionate and butyl acetate are effective baits for *Glischrochilus quadrisignatus* (Say), another economically important nitidulid. Attractive chemicals such as these are produced by the host plant or associated microorganisms. Curiously, no pheromones have been reported for nitidulid beetles, even though attractants of this type would probably be very useful in insect control, and pheromones are known to exist in a large number of other beetle species.

We now report an aggregation pheromone in *C. hemipterus* and describe a wind-tunnel bioassay. Synergistic effects of the pheromone and host plant volatiles are also documented, and the chemical identification and synthesis of two novel pheromone components are presented.

METHODS AND MATERIALS

Beetles. C. hemipterus was reared on a pinto bean diet as described by Dowd (1987). The culture provided beetles for pheromone production and for bioassays. Insects used for pheromone production were separated by sex when 0–1 week old.

Bioassays. All bioassays were conducted in a wind-tunnel olfactometer that measured 0.60 × 0.60 m in cross section by 1.35 m long. The sides and top of the wind tunnel were Plexiglas, and the floor was plywood. The ends were covered with 30-mesh steel screen. Air was forced through the wind tunnel by an electric fan that was connected by a duct to the upwind end. Laminar flow was achieved by passing the air through several layers of cheesecloth mounted at the upwind screen, as described by Baker and Linn (1984). The linear air flow rate was 0.3 m/sec. The temperature was kept at 27°C; the relative humidity was not controlled but was in the range of 30–40%. The wind tunnel was lit by four 40-W fluorescent tubes mounted 10 cm above the top.

About 24 hr before bioassays were to begin, cultures containing a total of 200–400 beetles, 0–1 week old and of mixed sex, were placed in a fume hood

for 8 hr. During this time, the diet medium dried down to about 75% of its original volume. The beetles were then transferred to the wind tunnel and kept without food for an additional 16 hr. Lights and air flow were left off during this time but were turned on before beginning bioassays. Beetles treated in this way appeared healthy and usually were ready to respond to attractive baits within 1 hr after the wind-tunnel lights and fan had been turned on.

Test treatments were suspended from a horizontal wire 0.4 m above the floor of the wind tunnel, perpendicular to the air flow and 0.2 m from the upwind screen. Treatments were always tested in pairs, separated by 0.3 m. The numbers of flying beetles landing at each treatment during the test period were recorded. Initially, observation periods lasted several hours, but as a structured bioassay method evolved, the test periods were decreased to 5 min, then to 3 min. Tests were always replicated, and each bait was tested at both locations, to avoid any bias resulting from position effects. When comparisons among three or more treatments were desired, all possible two-way tests were run using a balanced incomplete block design. Tests were separated in time by 2–5 min.

In initial experiments, whole cultures were used as baits. These were held in 30-ml plastic cups and contained ca. 30 beetles. Each cup was covered with cloth, which allowed volatiles to escape. In one experiment, a second, open cup containing fresh diet was attached immediately below the bait cup, so that any attracted beetles would be able to feed and would remain at the bait, allowing them to be captured and sexed after the test.

Extracts or chromatographic fractions to be used as baits were applied to 7-cm circles of filter paper, which were folded into quarters and secured with a paper clip. Concentrations of test solutions were adjusted so that the application volume was in the range of 10–30 μl. Further details of specific tests are given with results.

Statistical Analysis. Data were transformed to the $\log(X + 1)$ scale before analysis to stabilize variance. Balanced incomplete block experiments involving comparisons among three or more treatments were analyzed by the method of Yates (1940). Paired t tests were used when only two treatments were compared.

Extraction. As a typical example, 300 male beetles, 9–12 days old, and the diet medium from the four rearing cups that held them were extracted by soaking in 100 ml of methylene chloride for 15 min. The extraction was repeated twice more, and the combined extracts filtered and dried over sodium sulfate. The extract was reduced in volume to 10 ml by rotary evaporation. Concentrations of extracts were calculated as beetle equivalents per milliliter, based on counts of beetles and extract volumes.

Volatile Collection. A 50-ml filtering flask was fitted with a cork, into which a Tenax trap was inserted. The Tenax trap was prepared from a 10 cm $\times$ 0.5 cm (ID) piece of soft glass tubing. A piece of brass screen (100 mesh)

was sealed into one end by heating. The tube was filled to a depth of 0.5 cm with Tenax porous polymer (60–80 mesh, Alltech, Deerfield, Illinois), which had been cleaned by extraction with hexane in a Soxhlet apparatus. A plug of glass wool was placed over the Tenax. About 15 ml of pinto bean diet was placed into the flask and the tip of the Tenax trap adjusted to about 1 cm above the diet. A vacuum was applied to the Tenax trap so that volatiles within the flask were drawn through the trap. A second Tenax trap was attached to the side arm of the flask to clean the air drawn into the flask. Approximately 100 male beetles 1–2 weeks old were added to the flask, and the air flow through the flask was adjusted to 50 ml/min. The flask was kept in an incubator at 27°C and 40% relative humidity. At this humidity the diet dried out slowly over a week. With the diet in this condition, the beetles remained active and healthy, but the growth of mold was retarded. The beetles received 14 hr of light each day. Eighteen such flasks were operated in the incubator at one time. Pheromone collections were quantified in terms of beetle-days, defined as the average amount of pheromone collected from one bettle in one day. Volatile collections were also made from female beetles and from diet medium without beetles.

To extract volatiles from the Tenax traps, each trap was back-flushed three times with 200 μl hexane. Before returning the trap to its flask, air was passed through the trap to evaporate residual solvent. Traps were extracted every two or three days.

Chromatography. Column chromatography on silica gel was used for all initial purifications. Columns were usually 5 cm × 0.5 cm, and these were adequate for extracts with 100 beetle equivalents, including diet medium. Before chromatography, the solvent was carefully removed from these samples under nitrogen and the samples taken up in hexane. Columns were eluted with two column volumes (2 ml) of these solvents: hexane; 5%, 10%, and 50% ether in hexane; and 10% methanol in methylene chloride. Each solvent was collected as a separate fraction. Larger columns were used for extracts with greater numbers of equivalents.

The rinses from the Tenax traps were also applied to silica gel columns; collections containing 3000 beetle-days did not overload a 5-cm × 0.5-cm column.

Silica gel containing 25% $AgNO_3$ was also used as a packing in open columns (5 cm × 0.5 cm). The samples were applied in hexane and the columns eluted sequentially with hexane; 5%, 10%, and 25% ether in hexane; and finally, with ether.

All chromatographic separations and syntheses were monitored by gas chromatography (GC), using a Varian 3700 gas chromatograph that was equipped with flame ionization detector, splitless injector for capillary columns, effluent splitter for preparative GC on a packed column, and effluent collector

(Brownlee and Silverstein, 1968). The gas chromatograph was interfaced to a Hewlett-Packard 3396A integrator. Two columns were used: The first was a 15-m × 0.25-mm (ID) DB-1 capillary with a 1.0-μm film thickness (J & W Scientific, Folsom, California). For many samples, this column was programmed from 100° to 200°C at 10°C/min, although lower starting temperatures or higher final temperatures were sometimes required. Beetle-derived samples were usually concentrated to 1–5% of the original volume by careful evaporation under N_2, so that the 1- to 2-μl injections would contain enough material to be easily detected (>1 ng/component). Concentrations of compounds in extracts and fractions were estimated by using integrator units; the integrator was calibrated using standard solutions of heptadecane in hexane. The other column, used for preparative GC, was a 2-m × 2-mm (ID) glass column, packed with 3% OV-101 on Chromosorb WHP 100/120 (Alltech).

Retention indices (I) relative to n-alkane standards were determined for the male-specific hydrocarbons. The DB-1 column was programmed from 100° to 200°C at 10°C/min, and the retention indices calculated by linear interpolation (Poole and Schuette, 1984).

High-performance liquid chromatography (HPLC) was conducted isocratically using a Waters Associates model 6000 pump and R401 refractometer detector. Two columns were used. The first was a 30-cm × 0.75-cm (ID) PLGEL 50A 10-μm size-exclusion column (Polymer Laboratories, Shropshire, U.K.) eluted with hexane. The other column was a 25-cm × 0.46-cm (ID) Lichrosorb Si60 silica column (5 μm particle size) (Alltech), coated with $AgNO_3$ as described by Heath and Sonnet (1980). This column was eluted with 25% toluene in hexane. The void volumes for the two columns were estimated to be 8 and 3.5 ml, respectively. The beetle-derived samples were not concentrated enough to be detected by the refractometer. Effluent was collected as 0.5- or 1-ml fractions, which were later analyzed by GC and bioassayed.

Spectroscopy. Mass spectra were obtained on a Finnigan 4535 quadrupole mass spectrometer. Sample introduction was always by GC [15-m × 0.25-mm (ID) DB-1 capillary with a 0.25-μm film thickness]. An ionizing potential of 70 eV was used for electron impact (EI) spectra. Isobutane was the reagent gas for chemical ionization (CI) spectra. NMR proton spectra were obtained on a Bruker 300-MHz instrument. Samples were dissolved in deuterobenzene, and shifts were calculated relative to tetramethylsilane. Nuclear Overhauser enhancements (NOEs) were measured for some samples, and the difference spectra were obtained as described by Sanders and Mersh (1982). Further experimental details are given with results. Ultraviolet spectra were taken with a Perkin Elmer Lambda 4B high-performance UV spectrophotometer. The solvent was hexane.

Hydrogenation. Saturated derivatives of male-derived hydrocarbons were

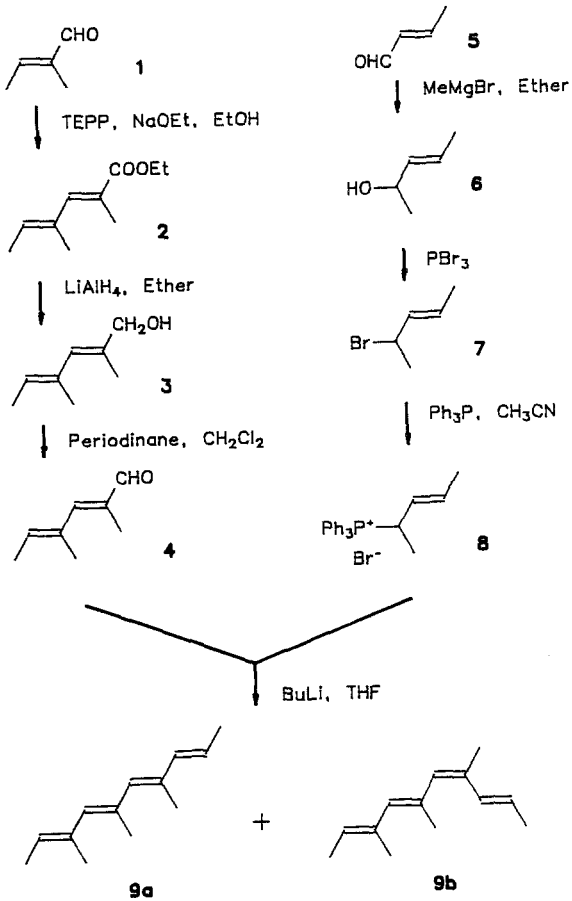

FIG. 1. Synthetic scheme for (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (*EEEE*-13, **9a**) and (2*E*,4*E*,6*Z*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (*EEZE*-13, **9b**). TEPP is an abbreviation for triethyl 2-phosphonopropionate.

prepared by the method of Parliment (1973), except that methylene chloride was used as the solvent. Palladium (10%) on carbon was used as the catalyst in the initial reactions, but PtO_2 was later found to be preferable.

Synthesis of 13-Carbon Tetraenes. (2*E*,4*E*,6*E*,8*E*)-3,5,7-Trimethyl-2,4,6,8-decatetraene (*EEEE*-13) and (2*E*,4*E*,6*Z*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (*EEZE*-13) were prepared as standards for comparison with the major male-specific compound from beetles. The synthesis is outlined in Figure 1. The reactions were monitored by GC and mass spectrometry. Except for the phosphonium salt, each intermediate product was used in the next reaction with-

out purification, other than drying over sodium sulfate and removal of solvent. Generally, each reaction produced a single major compound which, by GC, accounted for over 90% of the volatile reaction products. The exception was the final reaction, which produced the two stereoisomers, *EEEE*-13 and *EEZE*-13, in approximately equal proportions.

Tiglic aldehyde [**1,** 2-methyl-2(*E*)-butenal] was converted to the ethyl ester of acid **2** in a Wittig-Horner condensation with triethyl 2-phosphonopropionate, by the procedure of Gallagher and Webb (1974). This reaction is primarily *E*-directed (Boutagy and Thomas, 1974), and by GC, only one isomer was observed. Ester **2** was reduced with $LiAlH_4$ to alcohol **3** as described by Mori (1976) for a different ethyl ester. Alcohol **3** was then oxidized to aldehyde **4** with periodinane reagent (Dess and Martin, 1983). The other half of the target hydrocarbon was constructed by alkylating crotonaldehyde [**5,** 2(*E*)-butenal] with methylmagnesium bromide to form alcohol **6,** as described by Brooks and Snyder (1955), except that a commercially prepared Grignard reagent was used. The alcohol **6** was converted to bromide **7** with PBr_3 by the procedure of Noller and Dinsmore (1943), except that bromide **7** was recovered by extraction with hexane rather than distillation. Allylic bromide **7** was treated with triphenylphosphine in refluxing acetonitrile to produce phosphonium salt **8.** This salt was crystallized by washing the product repeatedly with dry ether. Finally, aldehyde **4** and phosphonium salt **8** were linked in a Wittig reaction (Sonnet, 1974) to form isomers **9a** (*EEEE*-13) and **9b** (*EEZE*-13) of the conjugated tetraene. Initial purification was on silica gel (elution with hexane). There appeared to be some decomposition on this column (formation of yellow color, which remained on the column), but both isomers were recovered.

One tetraene was thermally labile. On DB-1 (100–200°C at 10°C/min), it produced sharp peaks at 3.99 min (I = 12.06) and 5.47 min (I = 13.26), with a broad hump between these peaks. The initial peak, due to rearrangement in the injector, could be eliminated by cooling the injector temperature to 100°C, and the hump (which indicated on-column thermal rearrangement) could be eliminated by using a thinner film column (0.25 μm versus 1.0 μm), which allowed the compound to elute at a cooler temperature (ca. 115°C versus 155°C). Thus, I = 13.26 appeared to represent the intact compound. This compound was assigned the structure, *EEZE*-13, because other conjugated systems with internal *Z* double bonds have been known to undergo rearrangement at GC temperatures or lower (for example, Näf et al., 1975; Huisgen et al., 1967). The other synthetic isomer was assigned the structure, *EEEE*-13, and it was stable under capillary GC conditions (I = 13.83).

In order to confirm assignment of structures to the synthetic compounds, *EEEE*-13 was prepared by a second method, which formed the 6(*E*) double bond stereoselectively (Boutagy and Thomas, 1974). Aldehyde **4** (Figure 1) was subjected to a second Wittig-Horner reaction with triethyl 2-phosphonopro-

pionate, again followed by reduction to the alcohol with $LiAlH_4$ and oxidation to the aldehyde with periodinane. The resulting aldehyde, (2*E*,4*E*,6*E*)-2,4,6-trimethyl-2,4,6-octatrienal, was then coupled in a Wittig reaction with ethyltriphenylphosphonium bromide. The major product had GC retention index, I = 13.83, and the other isomer in the first synthesis (I = 13.26) was not detected at all. Thus, I = 13.83 did correspond to *EEEE*-13.

EEZE-13 and *EEEE*-13 could be completely resolved by HPLC on the $AgNO_3$ column. *EEZE*-13 eluted at 5.5 ml after injection, and *EEEE*-13 at 6.5 ml. Collected peaks were uncontaminated by the other isomer, and purities of the separated isomers exceeded 98%, by capillary GC. The concentration of *EEEE*-13 was determined by GC, using heptadecane as a quantitative internal standard, and an aliquot was diluted to 1 ng/10 μl for bioassay.

The following NMR data were obtained for *EEZE*-13: δ7.00 (1H, dqd, J = 15.7, ~1, ~1), 6.12 (1H, br s), 5.90 (1H, br s), 5.71 (1H, dqd, J = 15.7, 6.7, ~1), 5.55 (1H, qqd, J = 6.8, ~1, ~1), 1.98 (3H, br s), 1.93 (3H, d, J = 1.4), 1.74 (3H, br s), 1.72 [3H, dd (half concealed), J = 6.6, 1.6], 1.63 (3H, d, J = 6.8). The UV spectrum contained maxima at 224 nm (ϵ = 2.1×10^4) and 285 nm (ϵ = 2.1×10^4). The mass spectrum was indistinguishable from that of *EEEE*-13. The NMR, UV, and mass spectral data for *EEEE*-13 were identical to the insect-derived compound and are presented in the results section.

Synthesis of 14-Carbon Tetraenes. (2*E*,4*E*,6*E*,8*E*)-3,5,7-Trimethyl-2,4,6,8-undecatetraene (*EEEE*-14) and (2*E*,4*E*,6*Z*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (*EEZE*-14), were synthesized as in Figure 1, except that 2(*E*)-pentenal served as structure **5** in place of crotonaldehyde. Again, one isomer was labile under GC conditions (the intact compound eluting at I = 14.06), and by analogy to the 13-carbon tetraenes, it was assigned the structure *EEZE*-14. *EEEE*-14 was stable at GC temperatures (I = 14.76). These isomers were also separable by HPLC on the $AgNO_3$ column. Elution volumes for *EEZE*-14 and *EEEE*-14 were 5.4 and 6.3 ml, respectively. Purities were 85% and 98%, respectively. A bioassay solution of *EEEE*-14 was prepared as described above.

The following NMR data were obtained for *EEEE*-14:δ6.27 (1H, dq, J = 15.5, ~1), 6.06 (1H, br s), 6.04 (1H, br s), 5.71 (1H, dt, J = 15.5, 6.6), 5.53 (1H, qqd, J = 6.7, ~1, ~1), 2.12 (2H, qdd, J = 7.3, 6.6, ~1), 2.02 (3H, d, J = 1.3), 1.99 (3H, d, J = 1.1), 1.75 (3H, dq, J = ~1, ~1), 1.64 (3H, d, J = 6.8), 1.03 (3H, t, J = 7.4). The UV spectrum had maxima at 224 nm (ϵ = 1.1×10^4) and 287 nm (ϵ = 2.5×10^4). The mass spectrum was as in Figure 3 below (lower).

NMR data were also obtained for *EEZE*-14: δ7.03 (1H, dq, J = 15.7, ~1), 6.13 (1H, br s), 5.93 (1H, br s), 5.79 (1H, dt, J = 15.7, 6.6), 5.55 (1H, qqd, J = 6.9, ~1, ~1), 2.10 (2H, qdd, J = 7.4, 6.6, ~1), 1.98 (3H, d, J = ~1), 1.95 (3H, d, J = ~1), 1.74 (3H, br s), 1.63 (3H, d, J = 6.9), 0.99

(3H, t, $J = 7.4$). The UV spectrum had maxima at 225 nm ($\epsilon = 2.1 \times 10^4$) and 286 nm ($\epsilon = 2.1 \times 10^4$). The mass spectrum was indistinguishable from that of *EEEE*-14.

Chemicals. The compounds and reagents for chemical syntheses were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and were used as received for reactions. Solvents for syntheses were dried over 4 Å molecular sieves, except ether, which was dried over sodium metal.

RESULTS AND DISCUSSION

Evidence for Aggregation Pheromone. Development of a flight-oriented bioassay for attractants was the initial goal of this research. Because these insects are excellent fliers and, presumably, colonize new food sources primarily in this way, we believed a wind-tunnel assay would be more relevant to the ecology of the beetles than the more classical "pitfall" bioassay used for many stored-product beetles (Phillips and Burkholder, 1981; Oehlschlager et al., 1988). We observed very little flight when *C. hemipterus* beetles were transferred from cultures directly into the wind tunnel. Instead, they walked to the edges and corners and formed aggregations. After several hours, however, the aggregations dispersed and the beetles began to fly spontaneously. When a beetle culture (in a small cup) was hung in the upwind end of the wind tunnel, the flying beetles were moderately attracted to it. Responding beetles approached the culture from downwind, with a casting, hovering flight and eventually landed on the cup. These flights typically lasted 1–3 min and covered the entire length of the wind tunnel. This behavior was typical of long-range orientation to an odor source (Cardé, 1984). A cup with beetles and diet was visited more frequently than one with only diet medium; during 7 hr of observation, landings on the two types of baits were 97 and 2, respectively. This was the first indication that a pheromone was operating.

In a subsequent study, culture cups containing males and diet were far more attractive than ones containing females and diet. During 5 hr, 77 beetles landed on cups with males while only eight landed at cups with females. Both sexes responded to the baits, however. During two 16-hr periods, 142 beetles were collected after flying to cups containing males, and of the responding beetles, 44% were males. (During this time, the corresponding cups containing females attracted 24 beetles, of which 58% were males). Thus, there was evidence of a male-produced pheromone to which both sexes responded about equally.

A methylene-chloride extract of the culture cups with only males was more attractive in the bioassay than one derived from females. In 10 tests spanning a total of 80 min, 71 beetles landed on the male-derived bait, while only one

landed on the comparable female-derived bait (1 beetle equivalent per test). Thus, the pheromone could be extracted into solvent.

A more rapid bioassay response was needed so that the wind tunnel could be used to monitor pheromone isolation. We discovered that by starving the bioassay beetles for a longer period of time (e.g., 16 hr) responses to attractive baits in the wind tunnel would occur with greater frequency (e.g., >10 landings in a 5- or 3-min period). With these initial results, we began a more systematic investigation of the aggregation pheromone.

Pheromone Isolation. After column chromatography of the extract of male cultures on silica gel, none of the five fractions were active in the bioassay, compared with the original extract. However, the recombined fractions were highly attractive, indicating that the active compounds had eluted from the column but that more than one chemical was required for attraction.

We hypothesized that both male-derived and diet-derived volatiles were responsible for the activity of the culture cups. To identify which fraction of the male-derived extract contained the pheromone, we tested combinations of the five chromatographic fractions (hexane; 5%, 10%, and 50% ether–hexane; and 10% MeOH–CH_2Cl_2) in this way: In each combination, one of the fractions was derived from males and the remaining four, from females; all were used at 1 beetle equivalent per test. Each combination was tested against the whole extract of the female culture (the control in this experiment), also used at 1 beetle equivalent per test. Thus, all the bioassay treatments would contain the full complement of diet compounds as well as any "general" metabolites produced by beetles of both sexes. The combination of fractions would be expected to differ from the control only if the single male-derived fraction contained the pheromone. From Table 1, it is clear that the hexane fraction was the primary source of male-specific attractant(s). Furthermore, because one male-derived fraction was sufficient for the combination to be a potent attractant, the pheromone appeared not to include components of widely different polarity.

The diet-derived coattractant used to bioassay the male-derived silica gel fractions, although successful, was more elaborate than necessary. Table 2 demonstrates that the activity of the male-derived hydrocarbon fraction was greatly enhanced by a wide variety of food-related volatiles, not just by volatiles from the beetles' own cultures. Because reproduction occurs at feeding sites in these beetles, the enhanced attraction to combined host- and beetle-derived volatiles is undoubtedly of great ecological importance.

For consistency, we continued to use the female-derived diet extract as the coattractant in subsequent bioassays of chromatographic fractions; however, propyl acetate (10% in mineral oil) was found later to be equally effective. Because it was better defined and easier to prepare, it became the coattractant of choice in the later bioassays.

The active compound(s) from the male beetles appeared to have at least

TABLE 1. ACTIVITY OF SILICA GEL FRACTIONS OF MALE-DERIVED EXTRACT IN WIND TUNNEL

Male-derived fraction	Mean bioassay count ($N = 6$)	
	Fraction combination[a]	Control[b]
Hexane	23.3*	0.5
5% Ether–hexane	1.5	0.5
10% Ether–hexane	1.5	1.8
50% Ether–hexane	1.2	0.8
10% MeOH–CH_2Cl_2	2.2	1.3

[a] Each male-derived fraction was combined with the four complementary fractions derived from females (all at 1 beetle equivalent per test). *The hexane fraction was the only one to show significant activity compared with the control ($P < 0.01$, t test).
[b] The control for this experiment was the whole extract of a culture of females (1 beetle-equivalent per test).

one double bond because the 10% ether–hexane fraction from the $AgNO_3$ column contained most of the activity (Table 3). A hydrocarbon without double bonds would have eluted with hexane. Further purification by HPLC with the size-exclusion column yielded two consecutive 1-ml fractions that were quite

TABLE 2. ENHANCEMENT OF ACTIVITY OF MALE-DERIVED HYDROCARBONS BY VARIOUS COATTRACTANTS

Coattractant	Mean bioassay count[a]		
	Male-derived hydrocarbons[b]	Coattractant	Male H-Cs + coattractant
Extract of culture of female beetles	2.0 b	4.0 b	17.0 a
Apple juice	1.3 b	1.2 b	15.6 a
Banana + baker's yeast	3.5 b	2.2 b	30.4 a
Propyl acetate[c]	2.2 c	4.7 b	18.8 a
Ethanol[c]	4.5 b	0.4 c	32.1 a
Ethanol + acetaldehyde + ethyl acetate (1 : 1 : 1)[c]	4.5 c	9.2 b	29.6 a

[a] Each line of table is a balanced incomplete block experiment, in which the treatments were tested in pairs, in all possible combinations; $N > 8$ in each line except the first, for which $N = 4$. In each line, means followed by the same letter were not significantly different (LSD, 0.05).
[b] Hydrocarbons were extracted from a culture of male beetles and used at 1 beetle equivalent per test.
[c] Tested as 10% solutions or suspensions in mineral oil.

TABLE 3. ACTIVITY OF CHROMATOGRAPHIC FRACTIONS DERIVED FROM MALE *C. hemipterus* HYDROCARBONS.[a]

Fraction description	Mean bioassay count ($N = 4$)	
	Fraction + coattractant[b]	Coattractant[b]
$AgNO_3$ fractions (open column, from culture extract)		
Hexane	1.0	1.3
5% Ether–hexane	15.0*[c]	2.0
10% Ether–hexane	33.3*	1.3
25% Ether–hexane	6.7	2.5
Ether	1.3	2.0
Size-exclusion fractions (HPLC, from $AgNO_3$ 10% ether-hexane fraction, above)		
8–10 ml after injection	0.8	1.0
10–11 ml	12.0*	1.5
11–12 ml	9.3*	1.0
12–13 ml	3.0	1.0
13–14 ml	1.0	1.8
14–15 ml	1.0	1.3
15–16 ml	1.5	1.0
$AgNO_3$ fractions (HPLC, from Tenax collections)		
3.0–4.5 ml after injection	0.0	0.3
4.5–5.0 ml	0.0	0.0
5.0–5.5 ml	0.5	0.0
5.5–6.0 ml	12.8*	0.3
6.0–6.5 ml	12.0*	0.0
6.5–7.0 ml	25.8*	0.8
7.0–7.5 ml	4.8*	0.3
7.5–8.0 ml	0.8	0.3

[a] Hydrocarbons were isolated by column chromatography on silica gel prior to separations listed in the table. Fractions were used at 1 beetle equivalent per test (culture extract) or 3 beetle-days per test (Tenax collections).

[b] In first two experiments, coattractant was the extract from female beetles + diet; in the last experiment, coattractant was propyl acetate (10% in mineral oil, 10 μl per test).

[c] Active fractions indicated by (*); t tests, $P < 0.05$.

active (Table 3). Male-derived Tenax collections also provided active hydrocarbons, and these were fractionated by HPLC on the $AgNO_3$ column. Four consecutive 0.5-ml fractions had activity (Table 3). As with the separation on the open column, the retention of active fractions indicated unsaturation in the pheromone.

Parallel chromatographic fractions derived from female beetles were prepared, and the fractions from both sexes were analyzed by GC. In the active, male-derived HPLC fractions were at least 10 compounds that were absent from the females (Figure 2, Table 4). While the activity spanned four consecutive

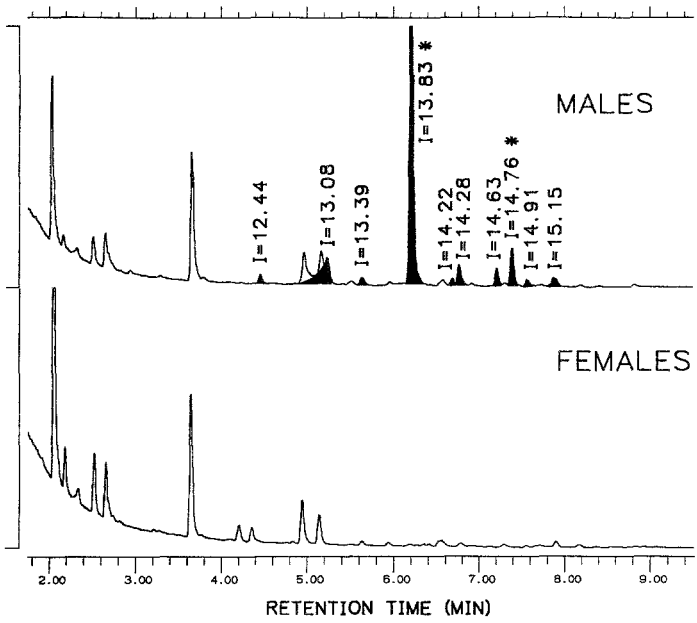

FIG. 2. Gas chromatograms of hydrocarbon fractions from male (upper) and female (lower) *C. hemipterus*. The samples were derived from Tenax collections. They were purified first by column chromatography on silica gel (elution with hexane) and then by HPLC on the $AgNO_3$ column (the GC traces represent the eluant between 5.5 and 7.5 ml). Structures of components indicated by (*) have been identified and proven by synthesis. Retention indices are listed above male-specific peaks.

$AgNO_3$–HPLC fractions (Table 3), no single compound was detected in more than two consecutive $AgNO_3$ fractions (Table 4). Thus, no single compound was absolutely required for activity and more than one subset of male-specific hydrocarbons was sufficient to elicit attraction in the wind tunnel. However, complete separation of these compounds was not obtained by any HPLC method. Preparative GC did not provide pure compounds either because many were too similar in GC retention or were too labile to survive this technique. Thus, it was not known whether all of the compounds in Table 4 were pheromonally active, and determination of the activity of individual compounds had to wait until synthetic compounds were available.

One difficulty in working with these compounds was that they were obtained only in minute amounts. In the extract of male cultures, 1 beetle equivalent contained approximately 1 ng of the major component (I = 13.83). In a typical Tenax collection, 1 beetle-day represented ca. 0.5 ng of this component. Because the beetles could live for several months in the aeration flasks, the

TABLE 4. MALE-SPECIFIC HYDROCARBONS IN *C. hemipterus*.[a]

Retention index (*I*) (DB-1 capillary GC column)	Approx. amount (pg) per male-equivalent	HPLC retention (ml)		Molecular weight	Formula
		Size exclusion	$AgNO_3$		
12.44	30	10.0-11.0*	6.0-6.5*	176	$C_{13}H_{20}$
13.08	200	10.5-11.5*	6.0-6.5*	176	$C_{13}H_{20}$
13.39	50	11.0-12.0*	5.0-5.5	176	$C_{13}H_{20}$
13.83	1000	11.0-12.0*	6.5-7.5*	176	$C_{13}H_{20}$
14.22	30	10.0-11.0*	5.5-6.5*	190	$C_{14}H_{22}$
14.28	50	10.5-11.5*	5.5-6.5*	190	$C_{14}H_{22}$
14.63	50	10.5-11.5*	6.5-7.0*	190	$C_{14}H_{22}$
14.76	100	11.0-12.0*	6.0-7.0*	190	$C_{14}H_{22}$
14.91	20	10.0-11.0*	6.0-6.5*	204	$C_{15}H_{24}$
15.15	40	10.0-11.0*	5.5-6.0*	204	$C_{15}H_{24}$

[a] Retention indices relative to *n*-alkanes. Presence of compounds in HPLC fractions determined by GC; many retention volumes represent two consecutive fractions which both contained the compound. Active HPLC fractions denoted by (*). Compounds with $I = 13.08$ and $I = 13.39$ also appear wherever the major component ($I = 13.83$) occurs; thus, they may be decomposition products. In Tenax collections, 1 beetle-day represents about 500 pg of the major component ($I = 13.83$).

Tenax collections were the richer source of active hydrocarbons, and furthermore, these were relatively easy to purify.

Mass Spectra. The EI mass spectrum of the most abundant compound ($I = 13.83$) is shown at the top in Figure 3. The apparent molecular weight, 176, was confirmed by the CI mass spectrum, in which the major peaks were 177 (M+H) and 233 (M+57, due to the isobutane reagent gas). The molecular weight is consistent with the formula, $C_{13}H_{20}$, indicating four double-bond or ring equivalents. The other male-specific peaks had similar fragmentation patterns, indicating hydrocarbons of 13, 14, or 15 carbons, all with four double-bond equivalents (Table 4). The EI spectrum for one minor component ($I = 14.76$) is also shown in Figure 3.

Hydrogenation of the Major Component. The saturated derivatives of the major component provided important structural information, but interpretation was complex. As shown in the gas chromatogram at the top of Figure 4, at least 12 distinct products were formed from the single parent compound. A typical mass spectrum of hydrogenation products is presented in Figure 3. The products were of two types that overlapped broadly: those with molecular weights of 182

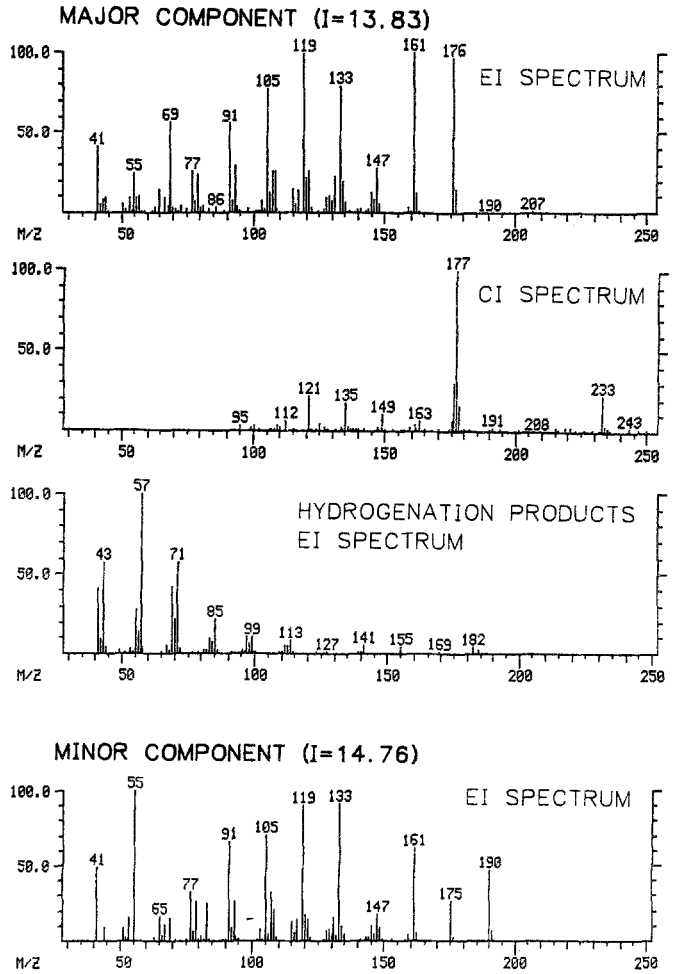

FIG. 3. Mass spectra of beetle-derived tetraenes and hydrogenated derivatives. The spectrum for the hydrogenated derivative contains peaks derived from both the cyclic (MW = 182) and acyclic products (MW = 184); see text.

and those with 184. The latter group was more informative. A molecular weight of 184 resulted from the uptake of eight hydrogens, indicating that the parent compound had four double bonds and no rings, if no triple bonds were present. (The compounds with molecular weights of 182 were also produced each time the reaction was run, and these never did hydrogenate further. Apparently, cyclic rearrangement competed with simple hydrogenation. PtO_2 as catalyst gave a

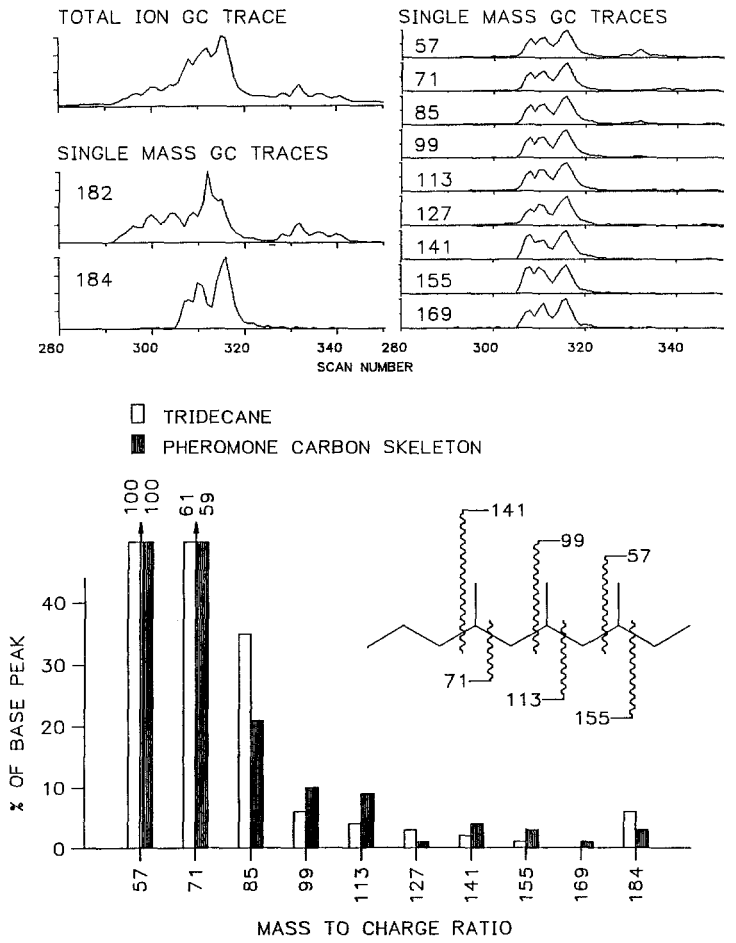

FIG. 4. Mass spectral analysis of hydrogenation products for major component (I = 13.83). Gas chromatogram of hydrogenation products is shown at top. These products had molecular weights of either 182 or 184 (second and third traces). Single mass GC traces for key fragment ions shown at upper right. GC temperature was increased at 10°/min; data acquired at the rate of 1 scan/sec. Fragmentation pattern compared with tridecane shown below.

greater proportion of the desired acyclic products than palladium did; thus PtO_2 was the catalyst of choice).

Both the number of distinct acyclic products (with molecular weight of 184) and their MS fragmentation patterns provided information about the parent compound. As shown in Figure 4, the GC trace for m/z 184 has three peaks.

The right-hand peak is broad and represents two compounds, which just began to separate; thus, there were apparently four acyclic saturated hydrocarbons derived from the parent compound. The retention indices for these three peaks were 11.50, 11.56, and 11.66. Being about 1.4 carbon units less than the *n*-alkane, these indices suggested a high degree of branching.

To make use of the MS fragmentation patterns, it had to be established whether the important fragments were derived specifically from the acyclic products. Key fragment ions in the mass spectra of acyclic saturated hydrocarbons are the series, m/z = 57, 71, 85, . . . , 169 (see Nelson, 1978). These correspond to $C_nH_{2n+1}^+$. GC traces for these masses, as well as for the molecular ions of the acyclic (184) and cyclic (182) hydrogenation products are given in Figure 4. Because the GC patterns for the key fragments followed that for 184 very closely, but not that for 182, these fragments were derived almost entirely from the acyclic products. Although the cyclic and acyclic products overlapped broadly in GC retention, the cyclic products were "transparent" and did not interfere with analysis of the $C_nH_{2n+1}^+$ fragments from the acyclic products.

Based on the $C_nH_{2n+1}^+$ fragments, all the acyclic products had nearly identical mass spectra. Compared with tridecane, the mass spectral peaks at m/z = 99, 113, 141, and 155 were relatively enhanced; while those at m/z = 85 and 127 were relatively suppressed (Figure 4). Interpretation of these data according to Nelson (1978) suggested 3,5,7-trimethyldecane as a likely structure. 3,5,7-Trimethyldecane has three asymmetric centers. If the original compound had double bonds involving the 3, 5, and 7 positions, then catalytic hydrogenation would create these asymmetric centers without stereoselectivity. The resulting eight optical isomers would produce, at most, four peaks on an achiral GC column, explaining the GC pattern we observed.

UV Spectrum of the Major Component. The UV spectrum of the natural material possessed a maximum at 287 nm ($\epsilon = 2.2 \times 10^4$) and another at 223 nm ($\epsilon = 1.0 \times 10^4$). The maximum at the longer wavelength suggested that three or four double bonds were in conjugation, but because steric and other factors can affect UV absorbance (Silverstein and Bassler, 1967), the exact number of conjugated double bonds was ambiguous.

NMR Spectrum of the Major Component. The NMR spectrum provided important structural information, but the lability of the compound made acquiring the data difficult. The initial NMR sample of about 20 μg was purified by preparative GC and transferred to a capillary NMR tube. The final purity of this sample was only 72%, however, by capillary GC, primarily because the compound had rearranged or decomposed to a significant extent on the preparative GC column. Nevertheless, the largest impurity was only 7% of the sample, so useful NMR data could still be obtained. A total of 30,000 scans were acquired. A subsequent NMR sample, containing about 30 μg, was prepared by HPLC on the size-exclusion column. After evaporating the hexane and add-

ing deuterobenzene, the sample was 90% pure, by capillary GC. A standard (5 mm) tube was used for this sample, and 3200 scans provided a reasonably good spectrum (Figure 5).

Spectral interpretation was complicated by sample degradation. The compound rearranged, polymerized, or both during acquisition of the spectra (in the latter sample, totally, within 10 hr). Peaks belonging to the original compound were distinguished from those due to decomposition by observing changes in the spectra over time. At first, no peaks were present in the region of 0.8–1.4 ppm, but over time, peaks in this area grew to become the dominant spectral features. Nevertheless, both NMR samples produced identical spectra when the peaks due to sample degradation were ignored. The observed resonances were: δ6.25 (1H, dq, J = 15.4, ~1), 6.03 (2H, br s), 5.63 (1H, dq, J = 15.4, 6.7), 5.53 (1H, qqd, J = 6.7, ~1, ~1), 2.00 (3H, br s), 1.98 (3H, br s), 1.74 (3H,

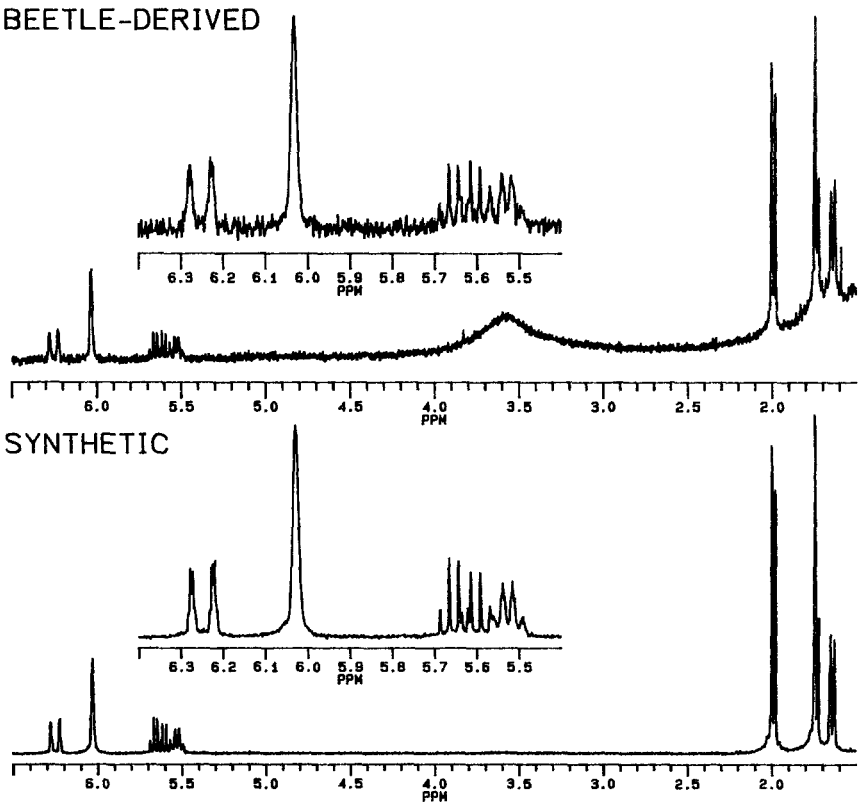

FIG. 5. Proton NMR spectrum of major beetle-derived tetraene (upper) and synthetic *EEEE*-13 (lower). Insets show expansion of olefinic region.

br s), 1.73 [3H, d (half concealed), $J = 6.7$], and 1.64 (3H, d, $J = 6.6$). All the resonances appeared to represent either olefinic protons or olefinic methyl groups. For five olefinic methyl groups to exist on the carbon skeleton in Figure 4, all four double bonds must be in conjugation (not just three, as suggested by the UV spectrum). Thus, the data suggested that the compound was a 3,5,7-trimethyl-2,4,6,8-decatetraene. The double bond at the 8 position had the *E* configuration because of the large coupling constant ($J = 15.4$ Hz) between the olefinic protons, but the configurations at the three trisubstituted double bonds could not be determined without having model compounds for comparison.

In retrospect, the observed sample degradation probably occurred because the NMR tubes had been prepared for use by heating in an oven at 110°C to drive off residual water. Synthetic tetraenes placed in similarly dried tubes also decomposed as noted above, but when the tubes were simply rinsed with hexane and dried under a stream of nitrogen, no decomposition of tetraene occurred.

Determination of Remaining Double-Bond Configurations of the Major Component by Synthesis. Once the model compounds were synthesized, it was clear that the unknown hydrocarbon was (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene (*EEEE*-13), because this hydrocarbon matched the beetle-derived compound in every way: GC retentions and mass spectra (of the hydrogenated derivatives as well as the parent compound), NMR spectrum, UV spectrum, and retention on the $AgNO_3$ and size-exclusion HPLC columns.

Identification of One 14-Carbon Minor Component. The mass spectrum of the minor component with $I = 14.76$ (Table 2) indicated a hydrocarbon with 14 carbon atoms and four degrees of unsaturation (Figure 3). Hydrogenation resulted in the uptake of eight hydrogen atoms, thus four double bonds were again indicated. Mass spectra of the hydrogenated products were interpreted as for the major component. Single-mass GC traces indicated that there were four products with molecular weight 198. These had retention indices of 12.52, 12.55, 12.62, and 12.63. The data suggested that the carbon skeleton was 3,5,7-trimethylundecane (Figure 6). Sufficient pure material was never available for an NMR spectrum of the original compound, but the similarity of retention on the $AgNO_3$ HPLC column suggested that this minor component and the major component had similar double bond systems. By analogy to the major component, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (*EEEE*-14) was chosen as the first synthetic target. Fortuitously, the synthetic compound matched the insect-derived component exactly, by GC, HPLC on the $AgNO_3$ column, MS, and by GC and MS of hydrogenated derivatives.

NOE Measurements. Nuclear Overhauser enhancement (NOE) was used to confirm in another way the unusual *EEEE* configurations of the pheromone components. The synthetic tetraenes, which were identical in all respects to the pheromone components and which were available in much larger quantities, made the NOE experiments possible. This NMR technique measures spatial

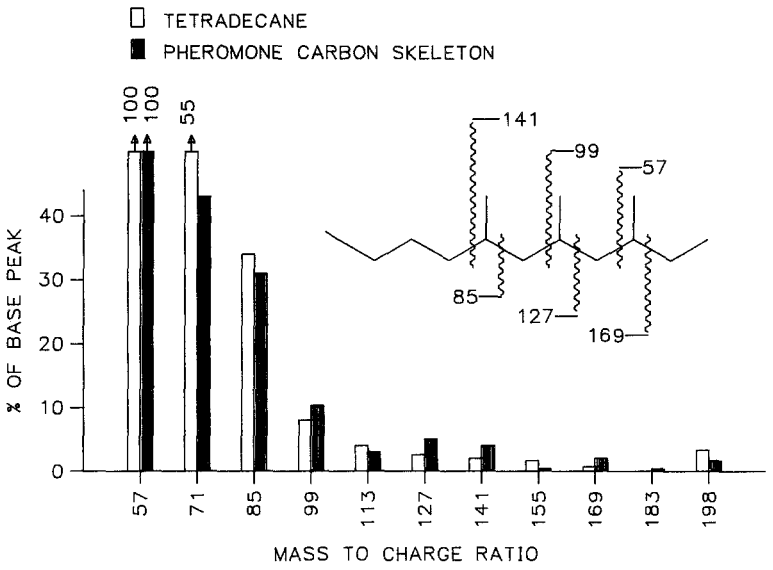

FIG. 6. Mass spectral analysis of the carbon skeleton of the 14-carbon minor component at $I = 14.76$.

relationships among protons and can determine the configurations of trisubstituted double bonds. Anet and Bourn (1965) reported a 17–18% enhancement of an olefinic proton signal when it was *cis* to an irradiated olefinic methyl group, but a *trans* proton experienced a decrease in signal intensity of 2–4%. Thus, by NOE, a clear-cut difference between *E* and *Z* double bonds in our synthetic tetraenes was expected. In order to conduct and interpret the NOE experiments, the proton shifts were first assigned (Figure 7), based on double irradiation experiments. (These assignments were later supported by NOE data).

With *EEEE*-14, irradiation at the δ1.75, 1.99, and 2.02 methyl signals produced NOEs of the protons at δ5.53, 6.04, and 6.06 of 0%, 0–2%, and 0–2%, respectively. The latter two NOE measurements were somewhat uncertain because the proton shifts were so close together, but all three values clearly supported the E configuration. Irradiation at δ2.02 did enhance the proton signal at δ5.71 by 9%. This result supported the 8(*E*) configuration because the 7 methyl group and the proton at the 9 position can be close in space only if the double bond at the 8 position is *E*.

Analysis of *EEEE*-13 was more complicated, because two proton resonances overlapped (δ6.03). When either the 5 or 7 methyl group was irradiated, a 2% enhancement of the δ6.03 peak was observed (4% if due solely to changes in one proton). These values were too small to indicate the presence of a *Z* double bond, but it was impossible to tell whether the enhanced proton was on the same double bond as the irradiated methyl group or on the adjacent one.

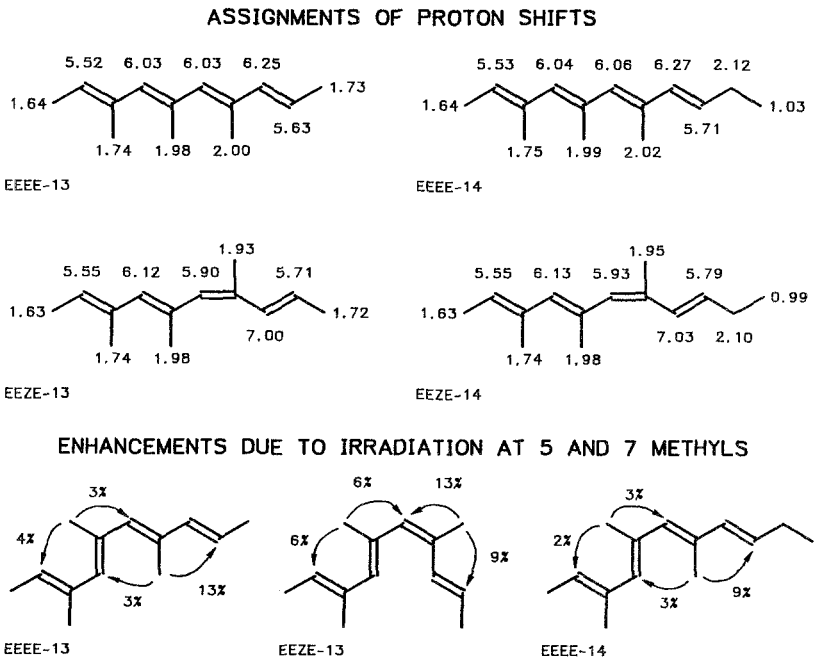

FIG. 7. Assignments of proton shifts for four synthetic tetraenes and NOE enhancements observed when the 5 and 7 methyl groups of three tetraenes were irradiated.

EEZE-13 was analyzed for comparison. Irradiation at the δ1.93 methyl signal caused a 13% enhancement of the proton signal at δ5.90, confirming the 6(*Z*) double bond, and irradiation at δ1.98 led to a 2% decrease in the signal at δ6.12, which supported the 4(*E*) configuration.

The major enhancements for these tetraenes following irradiation at the 5 and 7 methyl groups are shown in Figure 7 (lower). The NOE assignments for the protons at δ6.03 in *EEEE*-13 were made by analogy to *EEEE*-14. The NOE data suggested molecular conformations in benzene solution similar to those figured, but the double bonds were probably not coplanar. Molecular models indicated that a fully planar conformation would be strained and that in this conformation the enhancement of the proton at the 2 position should equal or exceed that at the 9 position. The enhancement of the proton in the 4 position would be similarly large in the *EEEE* tetraenes. Rotation about single bonds so that the 2, 4, and 6 double bonds were no longer coplanar would relieve the steric strain, would explain the fairly low observed NOE values, and may contribute to the unexpected maxima at 223–225 nm in the UV spectra.

Bioassays with Synthetic Hydrocarbon. Both *EEEE*-13 and *EEEE*-14 were active in the bioassay (Table 5). The major compound in the beetles, *EEEE*-13, showed significant synergistic activity with propyl acetate, although it was

TABLE 5. ACTIVITY OF SYNTHETIC HYDROCARBONS

Treatment[a]	Mean bioassay count[b]
Activity of *EEEE*-13 (N = 12)	
Control	0.1 c
EEEE-13 (1 ng)	0.5 bc
Propyl acetate (coattractant)	0.8 b
EEEE-13 (1 ng) + propyl acetate	5.7 a
Activity of *EEEE*-14 (N = 12)	
Control	0.1 c
EEEE-14 (1 ng)	1.8 b
Propyl acetate	2.0 b
EEEE-14 (1 ng) + propyl acetate	23.2 a
Comparative activities of *EEEE*-13 and *EEEE*-14 (N = 12)	
Propyl acetate	1.3 d
EEEE-13 (1 ng) + propyl acetate	4.7 c
EEEE-14 (200 pg) + propyl acetate	14.1 b
EEEE-13 + *EEEE*-14 (1 ng + 200 pg) + propyl acetate	25.0 a
Comparison of male-derived hydrocarbons with *EEEE*-13 (N = 8)	
Propyl acetate	0.4 c
Propyl acetate + *EEEE*-13 (1 ng)	5.5 b
Propyl acetate + male-derived H-Cs (1 ng of *EEEE*-13)	18.0 a
Comparions of male-derived H-Cs with combined *EEEE*-13 and EEEE-14 (N = 8)	
Propyl acetate	3.3 b
Propyl acetate + *EEEE*-13 + *EEEE*-14 (1 ng + 200 pg)	27.5 a
Propyl acetate + male-derived H-C's (1 ng of *EEEE*-13)	24.4 a
Synergistic activity of the *EEEE*-14 and a combination of ethanol, ethyl acetate, and acetaldehyde (N =12)	
Ethanol + ethyl acetate + acetaldehyde[c]	8.0 b
EEEE-14 (1 ng)	0.8 c
EEEE-14 (1 ng) + ethanol + ethyl acetate + acetaldehyde	31.2 a

[a] *EEEE*-13 = (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene; *EEEE*-14 = (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene; except where otherwise indicated, propyl acetate was the coattractant (used as 10% solution in mineral oil, 10 μl per test). Male-derived hydrocarbons were from Tenax collection, hexane fraction from silica gel. Amount tested contained 1 ng of *EEEE*-13.

[b] Each experiment was a balanced incomplete block with the treatments tested in pairs. Tests lasted 3 min. Analysis was in the log (X + 1) scale, conducted by the method of Yates (1940). Means were converted back to the numerical scale for presentation. In each of the six experiments, means followed by the same letter were not significantly different (LSD, 0.05).

[c] The three components were used in a 1:1:1 mixture as a 10% solution with mineral oil (see Smilanick et al., 1978).

not active by itself at the level corresponding to one male equivalent. The 14-carbon minor component, *EEEE*-14, was also synergistic with propyl acetate and, in addition, was significantly active by itself. Surprisingly, *EEEE*-14 was more active than *EEEE*-13, even when tested at one fifth the dose. The 14-carbon compound is a minor constituent in the male-derived hydrocarbons (about 10–20% as abundant as the major component), but it accounted for a large proportion of the pheromonal activity. Furthermore, these hydrocarbons synergized each other in much the same way as host-derived volatiles synergized the whole pheromone.

The fourth and fifth experiments in Table 5 demonstrate in another way that just the major 13-carbon component, *EEEE*-13, is insufficient for maximum activity. This compound attracted only about one third as many beetles as the whole, male-derived hydrocarbon mixture. However, when *EEEE*-13 and *EEEE*-14 were combined, the combination compared favorably with the male-derived attractant.

The final experiment in Table 5 shows the tremendous increase in activity that is possible by adding *EEEE*-14 to a previously reported "best" attractant (Smilanick et al., 1978).

Qualitatively, the two model compounds that do not occur in the beetles, *EEZE*-13 and *EEZE*-14, were not active in the wind tunnel. Bioassay details for these and other synthetic tetraenes will be published later along with the structures of the remaining male-specific hydrocarbons.

SUMMARY

This is the first report of an aggregation pheromone in a nitidulid species. A wind-tunnel bioassay was described that permits efficient monitoring of chromatographic fractions and evaluation of pure compounds. Two novel, conjugated tetraene hydrocarbon pheromone components were identified and synthesized. Insect pheromones with four conjugated double bonds have not been reported previously, although related compounds have been found in plants. For example, Boland et al. (1987) reported a series of 2,4,6,8-undecatetraenes in the marine alga *Giffordia mitchellae*, but these compounds do not have the methyl branches found in the *C. hemipterus* pheromone components. Interestingly, the cyclic mycotoxin, citreomontanin, from the fungus, *Penicillium pedemontanum*, contains the *EEEE*-13 structure as part of a side chain (Patel and Pattenden, 1985).

The pheromone was most active when used in combination with host-type volatiles. This enhancement is similar to that observed in other beetle groups (e.g., Walgenbach et al., 1987; Oehlschlager et al., 1988; Birch, 1984) and also in fly species that use aggregation pheromones (Bartelt et al., 1986). Fur-

ther research will explore the chemical nature of host-derived coattractants for *C. hemipterus*. The pheromone, used with the optimum coattractants, may become a useful pest management tool.

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RELEASE RATES OF TETRADECEN-1-OL ACETATES FROM POLYMERIC FORMULATIONS IN RELATION TO TEMPERATURE AND AIR VELOCITY

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Abstract—An apparatus was developed for investigating the release rates of pheromone formulations at temperatures and air velocities that correspond with those in the field. Polyurethane foam proved an excellent adsorbent for the recovery of tetradecen-1-ol acetates, allowing high air flows at low differential pressure and without breakthrough of these compounds. The experimental setup and procedures are described. The release rates of all formulations investigated were almost proportional to the square root of air velocity, and their logarithms were proportional to the reciprocal of temperature (K). When temperature is increased from 15°C to 25°C, the release rates increase by about 2–2.5×. Both relationships were combined into one equation that allows a reliable estimate to be made of the actual release rates of a formulation, under varying conditions, on the basis of its specific parameters and the relevant meteorological data.

Key Words—Air velocity, controlled release, formulation, pheromone, release rate, temperature, tetradecen-1-ol acetates, vapor collection.

INTRODUCTION

Synthetic sex pheromones can be used against lepidopterous pests in two different approaches: monitoring the pest insects using traps with the pheromone as a lure, and mating disruption, that is, reducing the chances of mating, either by attraction and removal of male moths from the population (male annihilation), or by disruption of the sexual communication. Reducing the chance of mating should prevent populations of pest insects building up to injurious levels (Kydonieus and Beroza, 1982; Campion, 1984).

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Devices that dispense pheromone to achieve direct control of insect pests need to fulfill more stringent requirements than lures used for monitoring the presence of a species. Monitoring is successful provided that substantial numbers of male moths are attracted if and whenever the species is present. For successful pest management by reducing mating, almost every moth of the population in question should be deprived of the opportunity to mate. This can only be achieved if the release of attractant or disruptant into the atmosphere is maintained on or above the effective threshold level during the whole period and all conditions during which the population reproduces. In communication disruption an underdose of the disruptant should never be tolerated, not even for a short period, as this might enable so many moths to mate that the delicate threshold for population control is exceeded. In male annihilation treatments, too, no drop of attractant release below threshold level can be afforded without risk of complete failure.

Since diffusion and air movement carry away the pheromone fumes from the treated areas, continuous replenishment is required. Therefore the disruptant or attractant should be formulated in such a way that the release rate ensures the minimum required dose under all circumstances in which mate-finding may occur. However, almost all formulations available for practical application do not release the agents at a constant rate. They show a decline of average release with time, in consequence of a complex of factors associated with aging (Zeoli et al., 1982; Daterman, 1982; Bierl-Leonhard, 1982). Much of the research on controlled release of pheromones has been devoted to this aspect, but release rates are also known to be influenced instantaneously by environmental factors such as temperature and wind, which may cause considerable fluctuations of actual release rates. It is precisely these actual release rates that determine the doses to which the target insects are exposed. Information on this actual exposure was required urgently for our studies on the working mechanism and feasibility of using the synthetic sex pheromone of the summerfruit tortrix moth (*Adoxophyes orana*, F.v.R.): Z9–14 : Ac and Z11–14 : Ac, 9 : 1, for communication disruption in this species. This information is essential in investigations into dose–response relationships, for interpreting the results obtained in the field, and for defining what specifications should be stipulated for formulations for practical use.

However, actual (unlike time-average) release rates cannot be measured in the field. Only more knowledge about the quantitative relationships between release rates and the variable environmental factors, together with relevant microclimatic recordings, would enable us to estimate fairly reliably the actual release rates of a dispenser and thus the doses in the field. Therefore, our main objective was to determine the relationships between release rates of the tetradecen-1-ol acetates Z9–14 : Ac and Z11–14 : Ac (TDA) from different formulations and the most relevant environmental factors: temperature and air

movement. Humidity was ignored, because its influence is likely to be of minor importance (Wiesner and Silk, 1982; Ioriatti et al., 1987).

It was decided to use the method of recovery and quantification of actually released TDA to determine the release rates, because other, possibly simpler methods, may be less reliable, as explained by Weatherston et al. (1981, 1982) and Bierl-Leonhardt (1982). Several different recovery methods have been reported in the literature (see Golub and Weatherston, 1984), but most of them allow for very restricted flow rates only. In cold trapping (Sower et al., 1971), solvent trapping (Beroza et al., 1975) and adsorption on glass wool (Pope et al., 1982), flow rates do not exceed a few milliliters per second. With adsorption on glass beads a flow rate of 17 ml/sec has been achieved (Golub et al., 1983), whereas with resin adsorbents (e.g., Tenax GC and Porapack Q) flow rates of up to 50 ml/sec have been applied (Qi and Burkholder, 1982). However, air velocities that match those in the moths' natural environment should be created through release chambers that are wide enough to contain current types of dispenser. To achieve this, flow rates of up to 1250 ml/sec would be required. Such rates could not be achieved with the adsorbents mentioned above, at least not without obtaining considerable breakthrough or having to scale up the recovery units to unpractical dimensions. Only Caro et al. (1977, 1978) have approximated such flow rates (2–3 m^3/hr). They used a pelletized form of molecular sieves as adsorbent for disparlure. This allowed such high flow rates, presumably because the air passes easily through the relatively wide spaces between the pellets. In such a system, however, a considerable amount of the volatized material may be expected to pass through without being adsorbed. The reported recovery rates vary between 75 and 100% (Caro et al., 1978). For our purpose, a more accurate quantification of the volatized compounds was required. After thorough investigation, we found porous polyurethane foam (PUF) (Turner and Glotfelty, 1977) to be an adequate adsorbent for TDA. It combined an outstanding adsorptive capacity with a very low air resistance, thus allowing a high flow rate at a low differential pressure and without breakthrough of the pheromone compounds. In this paper the experimental setup and procedure for our release rate measurements are described, and the results for some different dispensers are presented. Furthermore, the general relationships between release rate and temperature and air velocity are derived and discussed.

METHODS AND MATERIALS

Dispensers. The different dispensers are specified in Table 1. TDA of >99% isomeric purity was used, with 7% antioxidant (BHT) added. Types 1–3 were prepared by sucking the TDA into long tubes of low-density polythene (Talas, prod. No. 61550 and 63440) and leaving it for about 24 hr to enable

TABLE 1. FORMULATIONS AND THEIR SPECIFICATIONS.

Dispenser	length (mm)	OD (mm)	ID (mm)	Content (mg)	Surface area (cm^2)
1. Polythene tube, one end closed	17	1.5	1.0	14	0.80
2. Polythene tube, both ends closed	35	1.5	1.0	29	1.65
3. Polythene tube, one end closed	35	1.45	0.75	18	1.59
4. Polythene sampling vial (Kartell)	33	8.0	6.5	1	4.15
5. Microporous polymer tube (Accurel)	50	1.7	0.9	50	2.83
6. Nylon tube, one end closed	7	2.1	1.5	15	0.018
7. Laminate polymer (Hercon)	25.4			44	13.60
8. Glass fiber filter disk		5.5		3	0.24

impregnation of the tube wall. Subsequently 3 × 8-mm aluminium open rivets were slipped on the tubes and tightened at predetermined interspaces. Finally, the resulting dispensers were separated. Type 4 consisted of 1 ml polythene sampling vials (Kartell, prod. No. 730); they were prepared by filling them with 1 mg TDA in 1 ml hexane and allowing the solvent to evaporate before closing the lid. In type 5 the TDA was flushed through the cavity of hollow Accurel fibers (AKZO, No. P78/16/1) and thus absorbed in the void space of the microporous wall. The fibers were then cut into the desired lengths. Type 6 dispensers were prepared much the same as types 1 and 3, except that medical-quality nylon tube (Talas, prod. No. N4) and 3 × 16-mm rivets were used, and the viscosity of the TDA was first increased by adding 7% Cabosil M-S16 fumed silica gel to prevent loss by spillage from the relatively wide opening of the dispensers. No time was allowed for impregnation, because the nylon is impermeable to TDA. Type 7 was a Hercon (H) Luretape TM dispenser (Herculite Products, Inc., lot No. A00522). Dispensers of all these types are or have been used in the field on a large scale, either as lure or for communication disruption. Type 8 consisted of a disk of glass fiber paper, supported on a cover glass and oversaturated with TDA. Types 2, 4, 5, and 7 release the TDA exclusively through the polymer walls, whereas in types 1 and 3 the TDA is also released through the opening; presumably the release from types 6 and 8 corresponds to that from free liquid.

Release Unit. The release units (Figure 1A and B) comprise two release chambers of different size: the larger for low and moderate air velocities, the smaller for higher ones. The larger chamber (A) consists of a glass tube of 28 mm ID and 200 mm length, provided with PTFE protected sliding screw-thread joints (Sovirel No. 701.43) on both ends. One end is connected to the air-inlet manifold (V) and provided with a small plug of PUF (W), to smooth the air

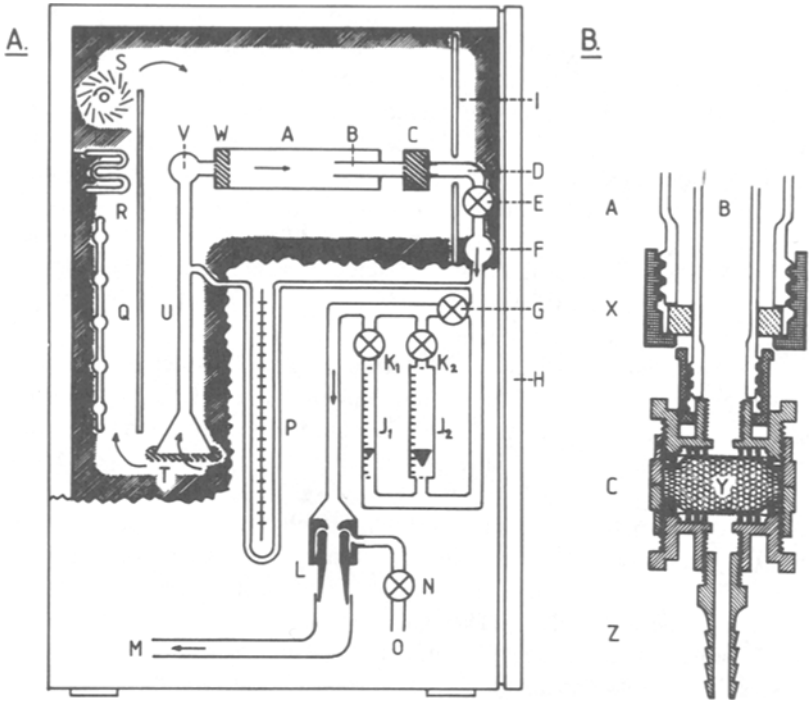

FIG. 1. (A) Schematic view of the release/recovery system (side wall of the constant temperature cabinet partly cut away to show internal parts). (B) Schematic view of recovery unit (longitudinal section). A: larger release chamber; B: smaller release chamber; C: vapor collector; D: flexible tubing; E: stopcock; F: outlet manifold G: stopcock for bypassing flowmeters; H: front of cabinet; I: screen, shielding the inner chamber when H is opened for access to E; J_1, J_2: flowmeters; K_1, K_2: stopcocks; L: air mover; M: air exhaust; N: pressure-reducing valve; O: mains compressed air; P: U-tube water gauge (glass, 75 cm); Q: cooler; R: heater; S: fan; T: polyurethane foam filter; U and V: copper inlet pipe and manifold; W: air-smoothing foam filter; X: sliding screw-thread joint; Y: polyurethane foam; Z: hose nipple.

stream. The other end is connected to the smaller release chamber (B): a glass tube of 12 mm ID and 100 mm length, with one screw-thread joint (Schott No. 29.227.06) linking it to the vapor collector (C). The dispensers are suspended in the center of the release chambers by a stainless-steel wire (0.25 mm diam.) clip, parallel with the air flow and not touching the walls. The open ends of types 1, 3, and 6 pointed downwind.

Smoke was used to reveal the pattern of the airflow in the release chambers. The pattern appeared to be very laminar and such as to prevent any vapor

from the dispenser from reaching either the inner wall of the larger release chamber, or the outer wall of the smaller release chamber.

Recovery Unit. Each recovery unit (Figure 1B) consists of a vapor collector (C) and includes a smaller release chamber (B). The collector was assembled by welding two open delrin filter holders face to face (Gelman, prod. No. 1107). A screwcap was glued on the inlet side to allow for connection with the smaller release chamber; the outlet side was provided with a hose nipple adapter (Gelman, prod. No. 73179) (Z). A PUF disk of 15 × 32 mm OD (Y) was introduced between the stainless-steel filter support screens. Eight release/recovery units are connected in parallel to an inlet manifold (V) and with flexible tubing (D) to stopcocks (E) on an outlet manifold (F).

Adsorbent. Pheromone released from a dispenser is recovered from the airstream by adsorption on PUF: porous polyurethane foam, polyether type, density 38, and colored grey by carbon (Uxem, 38HH). By comparing the difference between initial and residual amounts of TDA in type 8 dispensers with those recovered by the PUF, and by use of a secondary filter, the adsorptive efficiency of this material for TDA at high air velocity was found to be 98%. New PUF filter disks were pretreated by washing with 500 ml of acetone and 500 ml of hexane, in alternate aliquots of 20 ml, to remove compounds that interfere with the GC analysis. The same disks could be used several times, if cleaned with 20 ml acetone and 20 ml hexane before reuse.

Airflow control. The air flow in the release chambers is induced by a Jetflow Airmover J20E (Haskel, prod. No. 27020) (L), powered by mains compressed air (O) (Figure 1A). The rate of airflow depends on the resistance of the system and the suction power of the air mover. The latter depends on the pressure of the compressed air, which is controlled by a pressure-reducing valve (N). A filter of PUF (T) was mounted in front of the inlet manifold of the release units to clean the aspirated air. Behind the outlet manifold (F), two flowmeters (J1,J2) of different capacity (max. 40 and 650 ml/sec) were incorporated, which may be brought into operation by means of stopcocks (K1,K2). The differential pressure of the release/recovery units is measured by an U-tube water gauge (P).

Temperature Control. Because of its low heat capacity, air very quickly attains the temperature of its surroundings. Therefore most of the air system was fitted into a constant-temperature cabinet (Figure 1A). A fan (S) mixes the air continuously and forces it along the cooler (Q), the heater (R), and the Pt sensor of the controlling thermostat. The copper inlet pipe (U) and manifold (V) adopt and maintain the average temperature in the cabinet. By heat exchange, they reduce the fluctuation in the temperature of the air that passes through them from $\pm 2°C$ to less than $\pm 0.2°C$ at all air velocities.

Procedure. To achieve the desired air velocity in a release chamber, the corresponding flow is computed (cross-section of chamber × air velocity). With

one unit and the most adequate flowmeter in line (stopcock G off), the desired flow is adjusted by means of the control valve on the mains compressed air. The differential pressure over the unit is read from the water gauge. Subsequently, the flow in each separate unit at the same differential pressure is determined by turning off all other units and adjusting the differential pressure to the predetermined value by the compressed-air control valve. The flowmeters are then bypassed by turning on stopcock G, because their resistance would drastically reduce the air mover's capacity. When all units to be used have been turned on and the differential pressure has been readjusted to the predetermined value, then the flow through each unit is restored to the values determined earlier. The corresponding air velocities are computed by dividing these flows by the cross-section of the chambers.

Before first use, the dispensers were exposed to moderate air movement at room temperature in a fume hood for seven days, because in the first period of release, the release rate might decline disproportionately (Rothschild, 1979; Daterman, 1982). Between measurements, they were stored at 4°C in closed Petri dishes. Before being introduced into the release chambers, they were exposed to the stipulated temperature for at least 15 min. When at least 50 μg TDA had been released (preliminary experiments showed that the time required for this ranged from 30 min to 12 hr, depending on flow rate and temperature), the airflow was stopped and the dispensers were removed. The recovery units (B + C) were disconnected and eluted with 20 ml distilled acetone. Pentadecan-1-ol acetate (PDA) was added as an internal standard, and this mixture was concentrated to ca. 50 μl by evaporation at 30°C with a microrotavapor. The amount of TDA was assessed by GC (Intersmat GC-16; column: glass, 2 m × 2 mm ID, 2% SE-30 on Chromosorb WHP 80/100; carrier: nitrogen, 1.8 bar; oven: 180°C; detector: FID, 210°C). The relationship between TDA/PDA ratios from 19 : 1 through 1 : 19 and the corresponding peak-height ratios on the chromatogram (P_{TDA}/P_{PDA}) had been assessed beforehand. This relationship could be described as: amount TDA = amount PDA ($0.077 + 0.728\ P_{TDA}/P_{PDA}$).

Recovery rates, as determined by comparing the amount of recovered TDA with the difference between initial content and residue of TDA in different dispensers, averaged 95%.

To measure release rates in still air, a dispenser was suspended in a glass tube, identical to a larger release chamber. Both ends of the tube were closed by plugs of PUF. After at least 24 hr, a puff of air was passed through the tube, so that any vaporous TDA would be adsorbed onto one of the plugs. Subsequently, both plugs and the inner tube wall were washed with acetone, and the TDA was quantified by GC as described above.

Measurement of Residue in Dispenser. Dispensers were cut into small pieces and extracted, by shaking for 18 hr, in 4 or 10 ml of acetone to which a

known amount of PDA had been added. The amount of TDA was determined by GC, as described above.

Release Rates. After preliminary investigations, three series of release rate measurements were carried out.

In Series I, two very different formulations (type 2 and 6) were compared. The release rates of four dispensers of each type were determined at four constant temperatures (10°, 15°, 20°, and 25°C) and for each temperature at a range of constant air velocities from 18 to 170 cm/sec and in still air. The eight dispensers were always treated simultaneously.

In series II, two dispensers of each of the different types 1–8 were studied at constant temperature (25°C) at air velocities ranging from 5 to 360 cm/sec and in still air.

In series III, single dispensers of types 1–7 were investigated three times, and of type 8 once, at constant air velocity of 150 cm/sec and constant temperatures of 8°, 10°, 15°, 20°, 25°, and 30°C.

The specific release rates of the dispensers declined during the investigations. Therefore, the sequence of different air velocities at each temperature in series I and II, and also the sequence of different temperatures in series III, was chosen in such a way that the effect of any decline in release rate with time would be compensated for, and thus would bias the results only minimally.

RESULTS

Relation between Release Rate and Air Velocity. The preliminary results (van der Kraan, 1984) and the results from series I and II showed that for each separate dispenser at constant temperature, $T(K)$, a plot of the release rate, R_v, (μg/hr), against air velocity, v (cm/sec), on double logarithmic scale, gave a linear relationship in agreement with the expression: $\log R_v = \log p_T + a \log v$ (p is a constant). The relation between R_v of a certain dispenser and v at temperature T, can therefore be expressed by

$$R_v = p_T v^a \tag{1}$$

The data reveal no relation between the regression coefficient a and temperature. So, the value of a depends solely on the dispenser. The value of p_T, however, depends on temperature too.

For series I, the regression of $\log R_v$ on $\log v$ was computed for each dispenser and temperature. From that regression, the release rate R_{150} at v = 150 cm/sec, an arbitrarily chosen but relevant air velocity, was estimated as an indication of the specific release level of the dispenser. The average R_{150} of the four dispensers of each type is given in Table 2. At equal temperatures, the regression coefficients of the four dispensers were always very similar, although

TABLE 2. REGRESSION OF RELEASE RATE ON AIR VELOCITY AT DIFFERENT TEMPERATURES: RESULTS OF SERIES I[a]

	Polythene tube (disp. type 1)				Nylon tube (disp. type 6)			
Temp. (°C)	a	R_{150} (μg/hr)	CI (95%) (% of R)	N	a	R_{150} (μg/hr)	CI (95%) (% of R)	N
10	0.54	1.60	12.7	23	0.47	0.80	25.3	24
15	0.50	3.46	4.3	27	0.60	1.57	14.5	22
20	0.42	6.55	8.5	21	0.39	1.61	15.4	29
25	0.47	14.76	6.4	32	0.38	4.39	5.2	42
Total	0.48		4.9	103	0.44		9.4	117

[a] a: regressison coefficient of log (release rate) on log (air velocity); R_{150}: release rate at air velocity = 150 cm/sec; CI (95%): 95% confidence interval of release rate at mean air velocity.

the individual release levels showed some variation. Therefore, the data from the four dispensers at each single temperature were pooled. To take account of the individual differences in release level, the data had been converted previously so that the average release rate per dispenser was unity. The results for the polythene dispensers are presented in Figure 2. The regression coefficients a are given in Table 2; the differences between their values are not significant ($\alpha = 0.05$). To express the variability of the data, the 95% confidence intervals

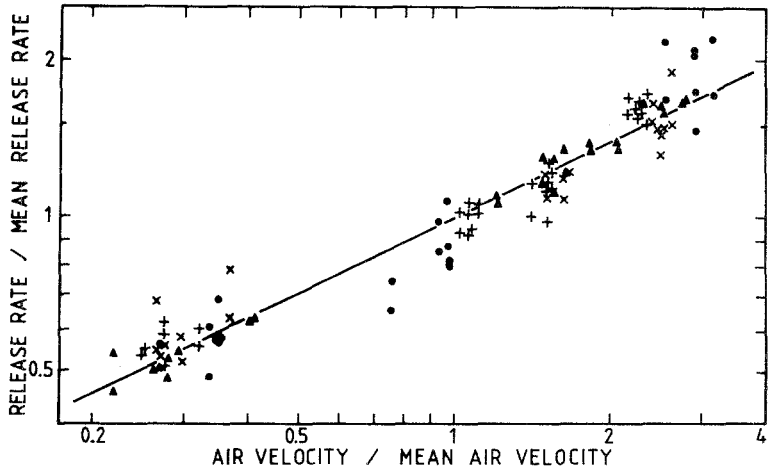

FIG. 2. Relation between release rate and air velocity for type 1 polythene dispensers of series I at 10°C (●), 15°C (▲), 20°C (x), and 25°C (+); line: regression line of log R on log v.

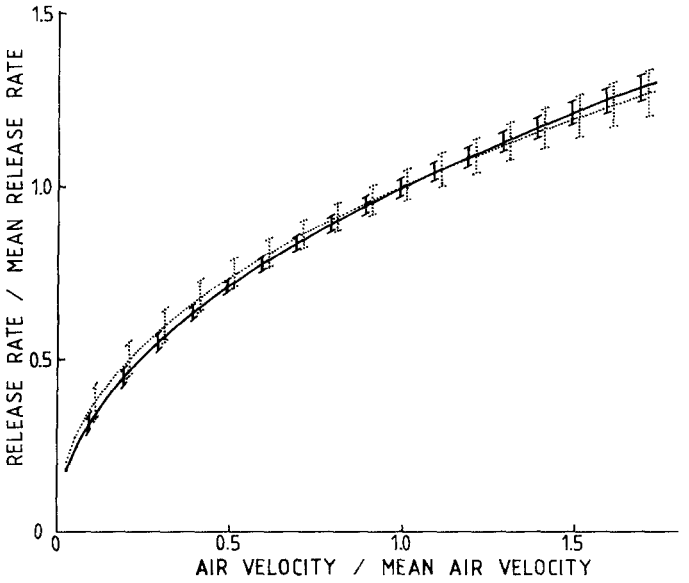

FIG. 3. Relation between release rate and air velocity: results of series I. Regression lines on linear scale of type 1 (——, polythene) and type 6 (. . . . . , nylon) dispensers. Vertical bars indicate width of 95% confidence intervals.

of the release rates were calculated (see Figure 3 for pooled data) and their width across the mean of the air velocities investigated is presented in Table 2 as a percentage of the corresponding release rates.

The data from all dispensers of series II were treated in a corresponding way. The regression lines are presented in Figure 4A and the values of a, R_{150}, and 95% confidence intervals in Table 3. The correlation coefficients for all dispensers were larger than 0.98. Although for some types of dispenser the values of a differ significantly ($\alpha = 0.05$), all differences are very small. The regression coefficient of the pooled data of all dispensers together is 0.50, and only types 2, 3, and 7 differ from this significantly.

The data on the release rates in still air from series I ($N = 52$) and II ($N = 15$) could not be worked out in the same way because of the log-transformation of v ($=0$). However, using the above-mentioned relation between R and v, the values of v that correspond with the experimental values of R were calculated, resulting in values between 0.1 and 0.4 cm/sec. This is very small compared with most air velocities that are relevant to the investigation, and therefore of negligible importance for the analysis and interpretation of the other data.

Relation between Release Rate and Temperature. The preliminary results

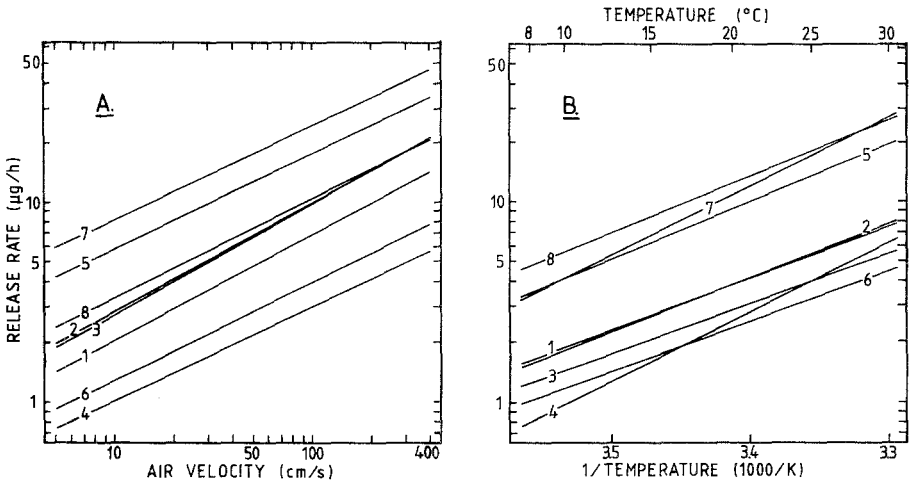

FIG. 4. (A) Relations between release rate and air velocity for different dispensers: results of series II. (B) Relations between release rate and temperature for different dispensers: results of series III. Dispenser types are numbered 1–8 as in Table 1.

and the results from series III revealed that for each separate dispenser at constant air velocity a plot of the logarithm of the release rate R_T against the reciprocal of the temperature T (K) gave a linear relationship, in agreement with the expression $\log R_T = \log q_v + b / T$ (q is a constant). However, a plot of R_T against T on double logarithmic scale also gave a linear relationship, in agree-

TABLE 3. REGRESSION OF RELEASE RATE ON AIR VELOCITY AT 25°C: RESULTS OF SERIES II[a]

Dispenser	a	R_{150} (µg/hr)	CI (95%) (% of R)	N
1	0.52 ab	8.37	8.7	36
2	0.54 ab	12.13	5.3	48
3	0.55 a	12.14	4.5	19
4	0.47 c	3.58	10.1	46
5	0.48 c	21.15	5.9	47
6	0.49 bc	4.75	11.6	22
7	0.47 c	29.04	2.8	15
8	0.50 abc	18.55	12.2	7
Total	0.50			240

[a]Legend as in Table 2. Values of a followed by the same letters are not significantly different.

ment with the expression: $\log R_T = \log s_v + c \log T$ (s is a constant). At air velocity v the relation between R_T of a certain dispenser and T can therefore be expressed by either

$$R_T = q_v 10^{b/T} \tag{2a}$$

or

$$R_T = s_v T^c \tag{2b}$$

The data reveal no relation between air velocity and the values of b and c; therefore it can be inferred that these values depend solely on dispenser type. The values of q_v and s_v, however, are influenced by air velocity too.

The data from the dispensers of each single type from series III were pooled before the linear regressions of $\log R_T$ on $1/T$ and on $\log T$ were computed. In all cases the correlation coefficients were well over 0.98, reflecting the excellent fit of the regression lines to the data. The regression lines of $\log R$ on $1 / T$ are presented in Figure 4B. The graph of those on $\log T$ was nearly identical and is therefore not shown. Data from series I cannot be used for the assessment of release–temperature relationships, as the data for different temperatures are substantially biased by the accompanied aging of the dispensers.

The values R_{25} of the release rate at 25°C, an arbitrarily chosen but relevant temperature, were estimated from the regressions as an indication of the specific release levels of the different dispensers. Their values, together with those of b, c, and the confidence intervals of R, are presented in Table 4. For

TABLE 4. REGRESSION OF RELEASE RATE ON TEMPERATURE AT 150 cm/sec: RESULTS OF SERIES III[a]

Dispenser	b	c	R_{25} (μg/hr)	CI (95%) (% R)	R_{25}/R_{15}	N
1	−2570	20.3 abc	5.34	4.9	2.00	18
2	−2680	21.2 ab	5.48	6.4	2.06	18
3	−2470	19.5 c	3.93	6.1	1.94	18
4	−3440	27.2 d	4.00	10.4	2.52	18
5	−2890	22.8 e	13.34	4.5	2.17	18
6	−2470	19.5 ac	3.24	7.7	1.94	16
7	−3470	27.3 d	17.08	10.6	2.54	18
8	2850	22.5 be	17.87	11.7	2.15	6
Total	−2860	22.6 b			2.16	130

[a] b: regression coefficient of log (release rate) on temperature^{-1}; c: ditto on log (temperature); R_{25}: release rate at 25°C; CI (95%): 95% confidence interval of release rate at mean temperature; R_{25}/R_{15}: quotient of release rates at 25°C and 15°C. Value of b and c followed by the same letters are not significantly different.

some of the dispenser types, the values of b and c differed significantly ($\alpha = 0.05$), irrespective of which of the two regression coefficients is taken into consideration (Table 4). To evaluate these differences, and as a suitable measure of the dependence of the release rate of the dispensers on temperature, the multiplication factors of the release rates at a temperature rise from 15°C to 25°C (R_{25} / R_{15}) were calculated. Results were nearly identical, regardless of whether equation 2a or equation 2b was used, and any difference was below the level of precision at which the values are presented (Table 4).

DISCUSSION

For air velocities from 5 up to at least 400 cm/sec, equation 1 can be used to express the relation between release rate R and air velocity v in all the dispensers investigated. The value of a averaged 0.5.

In still air ($v = 0$), we found that the release rate was not zero, but corresponded to the release as computed with equation 1 for air velocities of a few millimeters per second. Thus the role of molecular diffusion for transport of molecules from the boundary layer will be of the same magnitude as that of such minute air movements.

The significant differences found between values of a of some dispensers indicate that a may depend partly on dispenser type, although the range of variation is very narrow ($4.7 < a < 5.5$). The only property of the dispensers that interferes with airflow is shape: size and form may influence the flow pattern of the air along the surface and thus its effective velocity. This factor may account for the minor, although significant deviations of a from 0.5, found for some dispenser types. It can therefore be concluded that the release rate of TDA from our dispensers is approximately proportional to the square root of air velocity.

Wiesner and Silk (1982) investigated the release of Δ11-tetradecenal from different dispensers at air velocities of 4.5–170 cm/sec. The relation curve of their results also very closely approximates a square root function ($a = 0.49$). Bierl-Leonhardt et al. (1979) reported that the emission rate of disparlure from Hercon dispensers increased proportionately to airflow. Indeed, their data may be expressed by $R = 0.11 + 0.0034F$ μg/hr (F = flow rate, ml/min), suggesting a linear relationship between release and flow or, as v is proportional to F, between release and air velocity. However, their data are based on a very short range of flow rates ($50 < F < 150$ ml / min) and may be expressed equally well by $R = 0.016F^{0.73}$. These data are thus also fully compatible with the exponential relationship of equation 1, which seems generally valid.

When equation 1 is applied to evaporation rates of dichlorvos at different airflows (Gückel et al., 1973), a value for a of 0.42 is obtained. Both in this

case and the above-mentioned one of disparlure ($a = 0.73$), airflows correspond with air velocities of only a few millimeters per second. Under such conditions, molecular diffusion may be as important as air velocity in determining the release rate. Therefore the values of a, as derived from these data, are not sufficiently reliable to allow conclusions to be drawn about the relationship between release rate and higher air velocities or about the extent to which the value of a may depend on the nature of the volatile compound.

Air movement is assumed not to influence the properties of the dispenser itself, but to affect only the transport of vapor molecules from the boundary layer above the releasing surface. The main properties of the volatile compound that may be relevant to that process and might affect the value of a are diffusivity and vapor pressure.

Apart from the case of practically still air, molecular diffusion appeared to play an insignificant role in the transport of the molecules. Moreover, the variation in the diffusivity of most lepidopterous sex pheromones is restricted by the relatively homogeneous chemical composition and dimensions of their molecules. The variation in saturated vapor pressure is indeed much larger but does not exceed a factor of about 100 (see Hirooka and Suwanai, 1978; Olsson et al., 1983; Butler and McDonough, 1979, 1981). In our research, the release rates per unit of dispenser surface area, and thus the vapor pressures (Gückel et al., 1973), varied by a factor of more than 80 (cf. type 2 at 10°C and type 8 at 25°C). This, however, had no influence on the values of a. So, neither diffusivity nor vapor pressure of the pheromone will have a significant influence on the relation between air velocity and release rate. It can be expected, therefore, that the release rate of most pheromones from polymeric dispensers will be approximately proportional to the square root of the air velocity.

This may be of special importance for theoretical studies on dispersal and airborne concentrations of pheromones, as the equations used in such studies (Bossert and Wilson, 1963; Aylor, 1976; Hirooka and Suwanai, 1976) are generally based on the assumption of steady release rates, independent of air velocity.

For all the dispensers investigated, the relation between release rate and temperatures from 8°C to 30°C may be expressed by equation 2a or equation 2b. The values of b or c differ significantly between some dispensers, indicating that they depend on dispenser properties; q and s are constants, depending on both air velocity and dispenser type.

The relationship between $1 / T$ and $\log T$ for the temperatures considered in this research is practically linear (for 10–40°C the difference between $\log T$ and $2.908–129.18 / T$ corresponds to less than 0.2°C). This explains the close correspondence of the regressions of R on $1 / T$ and on $\log T$, and hence the identical results of the computations of R_{25} / R_{15} and of the significance of differences obtained by using either equation 2a or 2b. Over a wider range of

temperatures, however, the difference between both equations will increase and the validity of one of them may decrease.

Log R for the liquid phase of different volatile compounds is proportional to log P (P = vapor pressure), which is linearly related to $1 / T$ (Gückel et al., 1973; Olsson et al., 1983). Such a relationship has also been reported for the evaporation rate of the pheromone analog lauryl acetate (Gaston et al., 1971) and is essentially the same as that of equation 2a. So, of our two empirical equations, 2a seems to be the more generally valid one.

The release rates per unit of surface area, and hence the actual vapor pressures, are very variable among our dispensers; under equal conditions this rate is over 10 times greater in the "free liquid" dispenser 8 than in the polythene dispenser 2. The differences in temperature dependence of the release rates are less obvious, but in a number of cases are significant. Expressed as R_{25} / R_{15} (Table 4), in our dispensers this temperature dependence varies between 1.9 and 2.6. These differences may be ascribed to properties of the dispenser material. Values of P_{25} / P_{15} of Z9-TDA, derived from measurements on saturated vapor pressure by Hirooka and Suwanai (1978) and Olsson et al. (1983), amount to 2.68 and 3.52 respectively. From data reported for a number of other pheromone compounds, values for R_{25} / R_{15} or P_{25} / P_{15} of between 2 and 3.5 could be derived: dodecen-1-ol acetates: 3.03 (for Arn dispensers; Rothschild, 1979) and 2.93 (Olsson et al., 1983); Δ11-tetradecenal: 2.75 (Wiesner and Silk, 1982); lauryl acetate from different substrates: 2.3–3.5 (Gaston et al., 1971); disparlure from Hercon dispenserss: 3.36 (Bierl et al., 1976) and 3.48 (Bierl-Leonhardt et al., 1979). The data presented by Olsson et al. (1983) indicate that the longer the chain of the pheromone type compound ($C_{10} \rightarrow C_{14}$), the greater the dependence on temperature (P_{25} / P_{15}: $2.7 \rightarrow 3.5$). So, for pheromone compounds with even longer chains, values of over 3.5 might be expected. From all this it appears legitimate to assume that for most lepidopteran pheromone compounds the dependence of the release rate on temperature, expressed by R_{25} / R_{15}, will vary between about 2 and 4.

For computation of actual release rates of dispensers from their release characteristics, together with data on temperature and air velocity, equations 1 and 2a may be combined to

$$R = kv^a 10^{b / T} \tag{3}$$

or, if the release rate R'' at air velocity v'' and temperature T'' is known, to

$$R = R''(v/v'')^a 10^{b/T - b/T''} \tag{4}$$

This complex relationship between release rate, air velocity, and temperature is depicted in Figure 5 for average values: $a = 0.50$ and $b = -2860$ ($R_{25} / R_{15} = 2.15$). This figure clearly reveals the relative importance of changes in temperature and air velocity for the release rate, depending on the initial conditions.

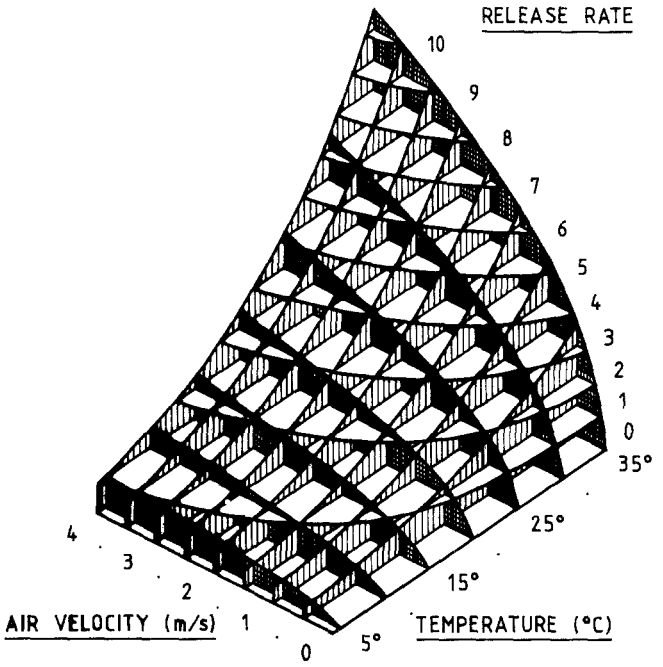

FIG. 5. Relation between release rate, air velocity, and temperature for $a = 0.5$ and $b = -2860$; release rate on relative scale.

It is generally assumed that among the environmental factors governing release rate from dispensing systems, temperature is more important than wind (Daterman, 1982). Our results indicate that slight differences in temperature, e.g., when determining release rates under laboratory conditions, may interfere less than changes in air velocity. This is especially true for low air velocities, because any changes will be relatively large then. As for the field, the mean daily temperature amplitude (difference between daily maximum and minimum temperature) from May through October in the Netherlands varies from about 5°C near the coast, up to 10°C inland (Anonymous, 1972). This would correspond with a variation in release rate of at most 2–4 times. The mean wind speed amplitude, on the other hand, is estimated at about 7 m/sec. Assuming that the release rate in still air equals that at about 4 mm/sec, this corresponds to a 40-fold range in release. Even taking into account that in a crop the amplitude of temperature may be larger, and that of wind speed smaller than under standard meteorological conditions, it will be clear that the effect of air movement should not be underestimated vis-à-vis with that of temperature. Thus, for dependable estimates of the fluctuating actual release rates of dispensers in the

field, accurate recordings of both air velocity and temperature at the proper spot, together with dispenser-specific release parameters, will be indispensable.

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ISOLATION AND IDENTIFICATION OF OVIPOSITION DETERRENTS TO CABBAGE BUTTERFLY, *Pieris rapae*, FROM *Erysimum cheiranthoides*

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Abstract—Avoidance of some crucifer species by the crucifer specialist, *Pieris rapae*, has been attributed to the presence of oviposition deterrents in these plants. Studies on one such unacceptable plant, *Erysimum cheiranthoides*, have resulted in the isolation of *n*-butanol-soluble deterrents from the alcoholic extract of foliage. The active fraction contained three cardiac glycosides, which were isolated by reversed-phase HPLC and by open column chromatography on silica gel. Chemical and spectral evidence (UV, [^{1}H]NMR, and FAB-MS) led to the characterization of these compounds as erysimoside (1), erychroside (2), and erycordin (3). Erysimoside and erychroside were strongly deterrent to *Pieris rapae*, but erycordin was inactive. Both active compounds have the same aglycone, strophanthidin (5) and the inner sugar in both cases is a 2,6-dideoxy hexose to which the outer sugar is attached at position C-4. These structural features, which are absent in the inactive compound (3), may represent specific requirements for oviposition deterrent activity.

Key Words—Lepidoptera, Pieridae, *Pieris rapae*, cabbage butterfly, *Erysimum cheiranthoides*, Cruciferae, oviposition deterrents, cardenolides, erysimoside, erychroside, erycordin.

INTRODUCTION

The cabbage butterfly, *Pieris rapae* L., oviposits on most, but not all members of the Cruciferae. Acceptance or rejection of a plant by gravid females depends on the presence of contact stimulants or deterrents, which can be extracted from

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the foliage (Renwick and Radke, 1983, 1985). One unacceptable crucifer, *Erysimum cheiranthoides*, is known to contain oviposition stimulants, but their effect is apparently blocked by potent deterrents (Renwick and Radke, 1987). Fractionation of alcoholic extracts of *E. cheiranthoides* foliage resulted in partitioning of the stimulants and deterrents between water and *n*-butanol. The *n*-butanol fraction was highly deterrent (Renwick and Radke, 1987). Subsequent chromatography of this *n*-butanol fraction has led to the isolation of active compounds, which have been characterized as cardenolides (Renwick et al., 1989). We now report on the complete isolation, identification, and bioassay of these compounds.

METHODS AND MATERIALS

Extraction and Fractionation of Plant Material. Erysimum cheiranthoides plants were grown in the greenhouse (Renwick et al., 1988) and harvested after three to four weeks. Fresh foliage (3.0 kg) was extracted in boiling ethanol for 5 min, cooled, homogenized, and filtered through glass wool. The alcoholic extract was evaporated to dryness under reduced pressure and then defatted with *n*-hexane. The defatted extract was dissolved in water (500 ml) and partitioned between water and *n*-butanol. The *n*-butanol fraction was concentrated under reduced pressure at $\sim 50°C$.

Instrumentation and General Techniques. UV spectra in methanol were recorded on a Perkin Elmer Lambda 5 UV/Vis spectrophotometer. All the positive-ion FAB mass spectra in thioglycerol matrix were acquired at Johns Hopkins University with a Kratos MS-50 operating at an accelerating voltage of 8 kV. The ionization was triggered by bombardment with xenon at 8 kV. For high resolution [^{1}H]-NMR spectra a Varian XL-400 instrument was used. Samples were dissolved in pyridine-d_5 and the spectra were recorded at 22–25°C. The chemical shifts were indirectly referenced to tetramethylsilane (TMS) by using signals of pyridine-d_5 as an internal reference.

Gas chromatography was conducted on a Perkin Elmer model 3920 equipped with a FID detector using a glass column (2 m × 2 mm ID) of 3% OV-101 on Chromosorb W-HP (100–120 mesh). The flow rate of carrier gas, N_2, was maintained at 40 ml/min and the injector and detector temperature at 280°C. The initial temperature was maintained at 100°C for 4 min and then programmed to 305°C at 8°C/min. For preliminary preparative HPLC, a reversed-phase C-18 Dynamax Macro HPLC column (25 × 2.1 cm, packed with irregular 8 μm particles, Rainin Instrument Co., Inc.) was used. The HPLC unit consisted of a Waters model U6K (Universal Liquid Chromatograph) Injec-

tor, 600 Multisolvent Delivery System, and Lambda-Max Model 481 LC spectrophotometer. For flash chromatography, Silica Woelm (32–63, um) was employed. Final purification was achieved by HPLC on a Varian 5000 Liquid Chromatograph using a reversed-phase Varian Micropak C-18 column (50 × 0.8 cm, irregular 10 μm particles). Thin-layer chromatography (TLC) was performed on precoated silica gel plates (10 × 5 cm, 250 μm thick, K6 silica gel, Whatman) or high-performance TLC (HPTLC) cellulose plates (10 × 10 cm, Whatman) using the following solvent systems: (1) $CHCl_3$–5% MeOH, (2) EtOAc–MeOH–H_2O (8:1:0.8), and (3) HCOOH–EtCOMe–t-BuOH–H_2O (15:30:40:15). The cardenolides were visualized by spraying with Kedde's reagent (Krebs et al., 1969) or a 1% solution of ceric sulfate in 2 N H_2SO_4 and sugars were visualized with aniline phthalate.

Isolation. The bioactive *n*-butanol fraction was chromatographed by preparative HPLC on the reversed-phase Dynamax column by loading 200 g (fresh weight) leaf equivalents (GLE) of the extract dissolved in 2 ml of water per injection. Elution was achieved by a water–acetonitrile gradient system at a flow rate of 15 ml/min, and the eluate was monitored at 254 nm. The fraction collected between 27.0 and 36.0 min was found to be most active. It was further resolved by flash chromatography using silica gel. The active fraction (2.1 kGLE) was loaded on a column of 20 g of silica gel packed in ethyl acetate saturated with water. The polarity of the eluent was gradually increased by adding methanol, and 24 fractions of 100 ml were collected. Compound **2** (Renwick et al., 1989) eluted in fractions 6–8 (4% methanol) followed by compound **1** in fractions 11–14 (7% methanol) and finally compound **3** in fractions 16–20 (10% methanol) (see Figure 1 for all compound structures).

Final purification of these compounds was accomplished by HPLC on a semipreparative reversed-phase C-18 column using a linear gradient of water and acetonitrile. The flow rate was maintained at 3.3 ml/min and the solvent composition increased from the initial 25% CH_3CN to 35% CH_3CN in 30 min. Under these conditions, compounds **1, 2,** and **3** had retention times of 14.6, 17.1, and 15.8 min, respectively. Surprisingly, the order of elution of these compounds is different on this column (Varian MCH-10, 50 × 0.8 cm) from that on the shorter column of the same packing material (Varian MCH-10, 30 × 0.8 cm). The order of elution of the second and third peaks in the active fraction (Figure 3 in Renwick et al., 1989) is reversed on the 50-cm column.

Bioassays. The activity of pure compounds as oviposition deterrents was tested on cabbage butterflies as previously described (Renwick et al., 1989). Five pairs of butterflies in each of six greenhouse cages were provided with a choice of two cabbage plants treated with a methanolic solution of test material or with solvent alone. A chromatographic sprayer was used to apply test materials evenly to all leaf surfaces. The eggs laid during a period from 1000 to

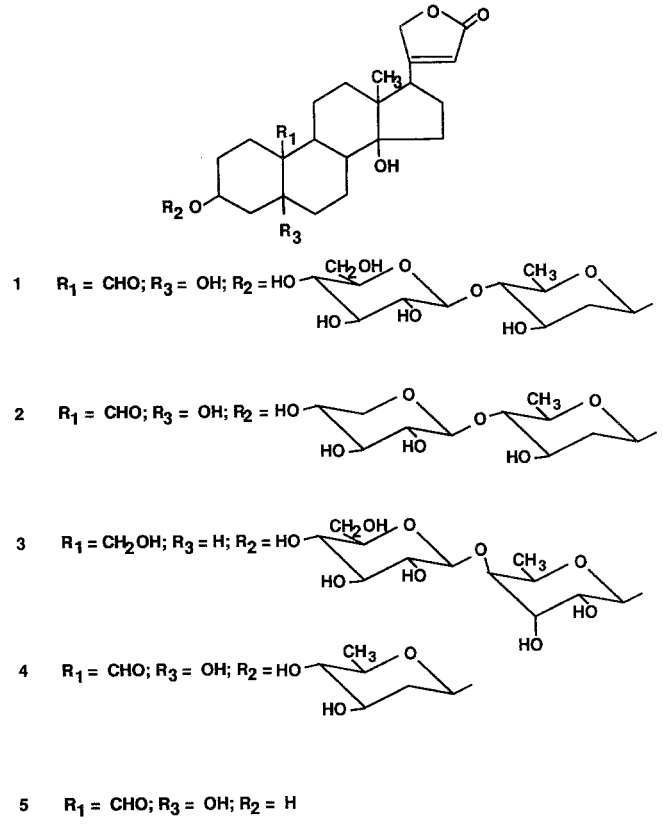

FIG. 1. Structures of compounds 1–5.

1500 hr were counted, and oviposition deterrent indices (ODI) were calculated according to the formula:

$$\text{ODI} = 100\ (C - T)/C + T$$

where C = number of eggs on control plant, and T = number of eggs on treated plant.

RESULTS AND DISCUSSION

Identification of Compound 1

Compound **1** was crystallized from methanol–ether as colorless needles, mp 210° C; UV λ_{max}: 216.3 nm (log ϵ 4.22). The UV spectrum indicated the presence of a butenolide ring in this compound. TLC on a silica gel plate in

solvent 2 gave a pink spot, typical for cardenolides at $R_f = 0.22$ with Kedde's reagent. A positive response to the Keller-Killiani test (von Euw and Reichstein, 1948) indicated the presence of a 2-deoxy sugar in this compound.

Mild Acid Hydrolysis of Compound 1. Compound **1** (2 mg) in 0.05 N HCl in 50% aqueous dioxane was heated at 50°C for 45 min. It was then diluted with water, dioxane was removed under reduced pressure, and the aqueous fraction was extracted with $CHCl_3$ (3 × 0.5 ml). The $CHCl_3$ fraction was concentrated, and the major product in this fraction was purified by HPLC on the reversed-phase semipreparative column. This compound was crystallized from aqueous methanol, mp 175°C, and it was identified as strophanthidin (5) by co-TLC and co-HPLC with the standard compound. The aqueous fraction was neutralized with Bio Rex[5] (CO_3^{2-} form) resin, and subsequent TLC in solvent 3 gave a spot at $R_f = 0.11$. This low R_f compared with that of glucose was indicative of a di- or trisaccharide.

Hydrolysis with 2 N HCl. Compound **1** (2 mg) dissolved in 2 N HCl in 50% aqueous dioxane was heated at 80°C for 1.5 hr. The resulting mixture was processed as before, and the $CHCl_3$ fraction thus obtained showed only a trace amount of strophanthidin along with several decomposition products on TLC in solvent 1. The aqueous fraction, however, showed two distinct spots at $R_f =$ 0.35 and 0.91. on a cellulose plate run twice in solvent 3. These spots corresponded to glucose and digitoxose respectively. The identity of these sugars was further confirmed by GLC of their TMS derivatives.

TMS Derivatives of Sugars of Compound 1. The aqueous fraction (100 μg) obtained by hydrolysis with 2 N HCl was stirred vigorously with Sil-Prep (Alltech) silylating reagent (100 μl) in an air-tight reaction vial for 5 min. It was then heated to 80°C for 20 min. Four microliters of this reaction mixture was injected into the GLC. Two peaks (corresponding to the α and β anomers) for digitoxose (R_t = 10.7 and 11.5 min) and two for glucose (R_t = 17.1 and 18.3 min) were obtained. TMS derivatives of authentic samples of digitoxose and glucose (Sigma) prepared in the same manner had identical retention times.

Enzyme Hydrolysis of Compound 1. Compound **1** (3 mg) in acetate buffer (pH 5.0) was incubated for 24 hr after the addition of β-glucosidase enzyme (from almonds, Sigma). The reaction mixture was then eluted through C-18 disposable columns (J. T. Baker, C-18 solid phase extraction columns, 3 ml, 40 μm) with water followed by methanol. The methanol fraction gave one spot on TLC. This product was identified by co-TLC, co-HPLC with the standard compound (Sigma), FAB-MS and [^{1}H]NMR (Tables 1 and 2) as helveticoside (**4**). The aqueous fraction was found to contain a sugar which was identified as glucose on the basis of TLC and GLC of the TMS derivative.

On the basis of the above results and its FAB-MS and [^{1}H]NMR spectra (Tables 1 and 2), compound **1** was identified as erysimoside, which has been previously reported from seeds of *E. cheiranthoides* (Makarevich and Kolesnikov, 1965).

TABLE 1. FAB MASS SPECTRAL DATA OF COMPOUNDS **1, 2, 3,** AND **4**

Fragment ion	*m/z* values			
	1	**2**	**3**	**4**
$[M+Na]^+$	719	689		557
$[M+H+Na]^+$			722	
$[M+Na-\text{outer sugar}]^+$	557	557		
$[M+Na-\text{outer sugar}-2H_2O]^+$			523	
$[Agl.+H]^+$	405	405	391	405
$[Agl.+H-H_2O]^+$	387	387	373	387
$[Agl.-H-H_2O]^+$	385	385	372	385
$[Agl.+H-2H_2O]^+$	369	369	355	369
$[Agl.-H-CO_2]^+$	359	359		359
$[Agl.+H-3H_2O]^+$	351	351	337	351
$[Agl.-H-H_2O-CO_2]^+$	341	341	327	341
$[Agl.-H-2H_2O-CO_2]^+$	323	323	309	323

Identification of Compound 2

Compound **2** was crystallized from methanol–ether, mp 245°C; UV λ_{max}: 216.3 nm (log ϵ 4.21); FAB-MS and [^{1}H]NMR data (Tables 1 and 2). Positive Kedde's and Keller-Killiani tests indicated a cardenolide with 2-deoxy sugar(s). Acid hydrolysis with 0.05 N HCl as for compound **1** produced strophanthidin (**5**) along with a saccharide unit. On heating with 2 N HCl for 1.5 hr, it gave traces of strophanthidin and two sugars which were identified as digitoxose and xylose on the basis of TLC using high-performance cellulose plates in solvent system 3. Double runs in this system gave two spots at R_f = 0.45 and 0.91, corresponding to xylose and digitoxose, respectively. For further confirmation TMS derivatives were made and compared with those of digitoxose (R_t = 10.7 and 11.5 min) and xylose (R_t = 14.4 and 15.3 min) on GLC. Enzyme hydrolysis of this compound with β-glucosidase yielded xylose (identified by TLC and GLC) and a monoglycoside. This monoglycoside was found to be identical with the enzyme-hydrolyzed product of compound **1**, i.e., helveticoside (**4**) on the basis of TLC, HPLC, mild acid hydrolysis, FAB-MS, and [^{1}H]NMR spectra.

These results led to the identification of this compound as erychroside (Makarevich and Kolesnikov, 1965).

Identification of Compound 3

Compound **3** was obtained as colorless crystals (aqueous MeOH); mp 204°C; UV λ_{max}: 216.8 nm (log ϵ 4.38); FAB-MS and [^{1}H]NMR data (Tables 1 and 2). A positive Kedde's test but negative Keller-Killiani test indicated that

TABLE 2. [^{1}H]NMR SPECTRAL DATA OF COMPOUNDS **1, 2, 3,** AND **4**[a]

Proton	**1**	**2**	**3**	**4**
3	4.31 bs	4.35 bs	4.35bs	4.36 bs
17	2.78 dd (3,8.5)	2.76 dd (4,8.5)	2.79 m	2.78 dd (4,8)
18	1.00 s	0.98 s	1.05 s	0.98 s
19	10.40 s	10.40 s	3.80 d(16) 4.04 d(16)	10.41 s
21a	5.08 d(18)	5.03 d(18)	5.03 d(18)	5.03 d(18)
21b	5.30 d(18)	5.29 d(18)	5.30 d(18)	5.23 d(18)
22	6.11 s	6.12 s	6.11 s	6.12 s
1′	5.38 bd(9.0)	5.40 bd(9.5)	5.36 d(9.0)	5.41 bd(9.5)
2′	1.85 m 2.25 m	1.87 m 2.28 m	4.56 m	1.90 m 2.29 m
3′	4.73 m	4.66 m	4.15 m	4.39 m
4′	3.68 dd (3,9.5)	3.66 dd (3,9.5)	4.35m	3.6 m
5′	4.40 m	4.37 m	4.56 m	4.27 m
6′	1.68 d(6)	1.65 d(6)	1.54 d(6)	1.56 d(7)
1″	5.00[b]	4.86 d(8.3)	5.00[b]	
2″	3.97m	3.88 dd(6,8.3)	3.98 m	
3″	4.24 m	4.12 m	4.15 m	
4″	4.24 m	4.12 m	4.95m	
5″	3.97 m	3.67 dd (9.5,11) 4.27 dd (5,11)	3.85 m	
6″a	4.40 m		4.35 m	
6″b	4.56m		4.56 m	

[a]Spectra recorded on 400 MHz in pyridine-d_5; chemical shifts given in ppm and J values in parentheses are in Hz; s = singlet; bs = broad singlet; d = doublet; dd = double doublet; bd = broad doublet; and m = unresolved multiplet.
[b]Hidden under water signal.

this compound is a cardenolide with no 2-deoxy sugar. The lack of this class of sugar was further confirmed by the fact that it remained unchanged on treatment with 0.05 N HCl. Compound **3** was subjected to hydrolysis with 2 N HCl under the conditions described for compound **1.** After the usual work-up, the organic layer showed three major and some minor spots on TLC (silica gel) in solvent system 2. The products could not be characterized because of insufficient material. The glycone part of compound **3** consisted of two sugars identified as glucose and 6-deoxygulose on the basis of TLC using high-performance cellulose plates in solvent system 3, which gave two spots at R_f = 0.35 and 0.68, respectively. The identities were confirmed by GLC of the TMS derivatives. The retention times of 13.1, 13.7, 17.1, and 18.3 min corresponded to

those of the TMS derivatives of 6-deoxy-D-gulose and glucose respectively. Despite the presence of a glucose unit, the original glycoside remained unchanged on treatment with β-glucoside. There are some reports of glucosides that are not cleaved by this enzyme (Rittel et al., 1952; Moore et al., 1954). The ease of enzyme hydrolysis thus appears to be dependent on the nature of the aglycone.

The results of hydrolytic experiments and comparison of the physical constants established the identity of compound **3** as erycordin (Makarevich and Kolesnikov, 1965).

In all three compounds, large J values of the signals for anomeric protons (Table 2) indicated that all the sugars in their $^4C_1$ conformation were linked by β-glycoside bonds.

Compounds **1, 2,** and **3** were tested for oviposition-deterrent activity. Helveticoside (**4**) and strophanthidin (**5**), which are the hydrolysis products of **1** and **2,** were also tested. The results (Table 3) show that the diglycosides of strophanthidin containing a 2,6-dideoxy sugar (i.e., **1** and **2**) are strongly deterrent. However, compound **3,** which is a diglycoside of cannogenol and lacks 2,6-dideoxy sugar, is not active. Since helveticoside (**4**) and strophanthidin (**5**) are also inactive, the presence of a strophanthidin nucleus and a 2,6-dideoxy sugar could be important, but the additional presence of a substituent on the 2,6-dideoxy sugar, e.g., glucose in erysimoside (**1**) and xylose in erychroside (**2**), seems to be essential.

Rothschild et al. (1988) have reported the presence of a strophanthidin diglycoside in *Cheiranthus allionii* as a contact oviposition deterrent to the large

TABLE 3. EFFECT OF CARDENOLIDES **1, 2, 3, 4,** AND **5** ON OVIPOSITION BY *Pieris rapae*

Compound[a]	Total number of eggs from 6 replications: Treated plants (T)	Total number of eggs from 6 replications: Control plants (C)	ODI[b]	$P \leq$ (paired t test)
Erysimoside (**1**)	138	461	53.9	0.01
Erychroside (**2**)	210	725	55.0	0.0005
Erycordin (**3**)	121	178	19.0	0.375
Helveticoside (**4**)	541	662	10.0	0.1
Strophanthidin (**5**)	587	583	−0.34	0.4

[a] Each tested at a concentration of 0.1 mg/plant.
[b] ODI = 100 $(C - T)/C + T$.

white butterfly, *Pieris brassicae*. In our general screening of some crucifers unacceptable to *Pieris rapae*, we found that erychroside (**2**) was the major cardenolide in the foliage of commercial wallflower var. cloth of gold (Thompson Morgan Seed Co.) as well as *C. allionii* supplied by Dr. Rothschild. This observation suggests that the compound isolated by Rothschild et al. (1988) is likely to be erychroside (**2**). It also suggests the existence of the same or a similar mechanism of contact oviposition deterrence for the two species.

Additional cardenolides isolated from *Erysimum cheiranthoides* as well as some commercially available cardenolides are now being studied to determine the relationship between structure and activity. Preliminary experiments indicate that larval feeding deterrents are also present in extracts of *E. cheiranthoides*, and future work will aim to elucidate the adaptive significance of both oviposition and feeding avoidance behavior of *P. rapae*.

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EXOCRINE SECRETIONS OF BEES
X. 3,7-Dimethyldeca-2,6-dien-1,10-diol: A Sex-Specific Compound from *Nomada annulata* (Hymenoptera: Anthophoridae)

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Abstract—3,7-Dimethyldeca-2,6-dien-1,10-diol was isolated from male cephalic extracts of the cleptoparasitic or "cuckoo" bee, *Nomada annulata*. The compound is absent in female head extracts. This diol, previously known only from a male danaid butterfly, is a new bee natural product and is not found in the volatile exocrine secretions of the host bee, *Andrena macra*. The role of this compound in this parasite–host system, including the chemical basis of *Nomada–Andrena* associations, is discussed.

Key Words—Cleptoparasite, *Nomada annulata*, Nomadinae, *Andrena macra*, Andrenidae, Hymenoptera, mandibular gland, male specific, diol, host parasite.

INTRODUCTION

There are approximately 5000 species of cleptoparasitic bees worldwide (Duffield et al., 1984), which include over 300 described species of *Nomada* in North America north of Mexico (Hurd, 1979). Fewer than 5% of these have been associated with their hosts. Known hosts include species belonging to the Andrenidae, Anthophoridae, Halictidae, and Melittidae. The reasons that spe-

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cific *Nomada* choose a particular host bee are poorly understood. Tengö and Bergström (1975, 1976, 1977) have investigated the chemical basis of this problem with several European cleptoparasite–host pairs of bees. In each case the chemical profile of the volatile components released from the male cephalic secretion of *Nomada* is similar to that of the Dufour's gland of the female host. For example, in two host–parasite pairs, *Melitta haemorrhoidalis* (Melittidae)–*Nomada flavopicta* and *M. leporina–N. flavopicta*, the secretions are dominated by octadecyl butanoate (Tengö and Bergström, 1976). The *Melitta* Dufour's glands exhibit more volatile components than the *Nomada* cephalic extracts. Similarly for the pairs *Andrena haemorrhoa* (Andrenidae)–*N. bifida* and *A. carantonica–N. marshamella*, there is a common major component, all-*trans*-farnesyl hexanoate. In the other two pairs, *A. helvola–N. panzeri* and *A. clarkella–N. leucophthalma*, the cephalic and Dufour's gland secretions are dominated by geranyl octanoate (Tengö and Bergström, 1977). These authors do point out that the male cephalic extracts of three additional species of *Nomada* do not correspond with the Dufour's gland chemistry of the host *Andrena*. In each species of *Nomada* investigated by Tengö and Bergström, male and female cephalic extracts contained completely different chemical components.

Tengö and Bergström (1977) postulated that during copulation, the volatile mandibular gland secretions of the male *Nomada* are sprayed onto the female partner. Gas chromatographic–mass spectroscopic analyses of extracts of females verified that the females had these chemicals after copulation. Tengö and Bergström (1977) speculated that chemical correspondence between the female *Nomada* and the host bee may allow the female *Nomada* entry into the host nest without being detected or repelled by the host female.

In a different approach to the study of host–parasite associations between *Andrena* and *Nomada* species, Cane (1983) tested the effects of 13 olfactory stimuli on the searching behaviors of the cleptoparasites. Artificial holes were bored in the ground to mimic the appearance of the host nests. Stimuli were placed in the holes. The searching behaviors of the *Nomada* were compared with those of the host *Andrena*. The experiments were run in Ithaca, New York, at a nesting site containing *Andrena alleghaniensis*, *A. regularis*, and the halictids *Agapostemon sericeus* and *Halictus ligatus*. The dominant *Nomada* at the site was *N. pseudops*. Some of the stimuli tested included: (1) chilled *Andrena* with pollen, (2) frozen *Nomada*, (3) peony anthers, (4) *Typhus* pollen, (5) *Andrena* Dufour's gland, and (6) chilled *Agapostemon* with pollen. Cane found that *Nomada* appeared to locate host nests primarily by visually searching for entrance holes. The searching *Nomada* most frequently entered artificial host nests containing *Andrena* with pollen.

In another chemical investigation of cleptoparasitic bees, Hefetz et al. (1982) found no similarities between the host bee *Calliopsis andreniformis* (Andrenidae) and its cleptoparasite, *Holcopasites calliopsidis* (Anthophoridae:

Nomadinae). The Dufour's secretions of the host bee are dominated by hydrocarbons. Males of *H. calliopsidis* were not analyzed.

During the past 10 years, we have been studying the biology of the solitary bee, *Andrena macra*, and one of its nest parasites, *Nomada annulata*. In an earlier investigation the chemistry of the Dufour's gland secretion of *Andrena macra* (reported as *Andrena flexa*) was reported (Fernandes et al., 1981). Here we report the chemistry of the male cephalic extracts of *Nomada annulata*.

METHODS AND MATERIALS

Collection of Bees. Specimens were collected by hand net at the Marine Training Base at Quantico, Virginia. The *Nomada* bees were collected during April and May, 1985–1987, at several *Andrena macra* nesting sites. Specimens were placed individually in glass shell vials and cooled in an ice chest for transport to the laboratory. Heads of specimens were removed with forceps and extracted with methylene chloride (100 heads/vial). Separate extracts were made of male and female specimens. One extract contained female heads and thoraces. An extract was prepared of 100 male *N. annulata* heads in 0.5 ml methylene chloride to be used for behavioral testing.

Chemical Analyses. The solvent was drawn off the head extracts, dried over Na_2SO_4, and concentrated by air evaporation. Extracts were analyzed on a Finnigan MAT 4500B gas chromatograph–mass spectrometer utilizing a 30-m × 0.25-mm capillary fused silica column containing SP-2100 (0.25 μm) temperature programmed from 60° to 300°C at 10°C/min. Retention times and mass spectra were compared with those of authentic compounds obtained commercially or synthesized by standard methods.

Proton and [^{13}C]NMR spectra were taken on a Varian 300 MHz spectrometer or a Bruker AM-300 spectrometer in deuterochloroform using tetramethylsilane as an internal standard.

Behavioral Tests. The field tests were run on May 14, 1988, between 10:00 AM and 2:00 PM. The day was sunny and the temperature rose during the test period from 68° to 75°F. There was little to no breeze. Both male and female *N. annulata* and female *Andrena* were active at the nesting site. This particular site contained over 1000 *A. macra* nests.

The procedure for the first series of tests consisted of placing filter paper squares (0.5 cm^2) on an insect pin and raised 2–3 cm above the ground. The test paper was coated with 5 μl of a standard solution containing 3,7-dimethyldeca-2,6-dien-1,10-diol (1 mg/ml methylene chloride) and allowed to stand for 1 min after the test solution was added. This allowed the methylene chloride to evaporate before running the behavioral tests. The test filter paper square was placed in the *A. macra* nesting site. All *N. annulata* passing within 12 in. of

the test filter paper were counted during the 10-min test interval. Controls consisted of filter paper squares treated with 5 μl of methylene chloride, mounted the same manner on an insect pin and allowed to evaporate for 1 min before each test was run. Data was reported in the same manner as described above. Each test was repeated three times.

In a second series of experimental tests, 25 μl of a methylene chloride extract of male *N. annulata* heads were added to the filter paper squares, allowed to evaporate for 1 min, and placed in the nest site. Data were reported in the same manner as cited above. The test was repeated three times. Controls were the same as those in the first experiment.

RESULTS

Chemical Analyses. Separate male and female *Nomada* head extracts were analyzed by combined gas chromatography–mass spectroscopy. Extracts from both males and females contained a series of saturated and unsaturated hydrocarbons including: tridecane (MW 184) (0.7%), heptadecane (MW 240) (2.5%), nonadecane (MW 268) (32%), heneicosane (MW 296) (3%), tricosane (MW 324) (7.5%), pentacosane (MW 352) (14%), heptacosene (MW 378) (3%), and hentriacontene (MW 434) (11.3%). In addition, both extracts showed hexadecanoic and octadecanoic acids in trace amounts. The male extract exhibited another component (26% of total volatiles) that eluted between heptadecane and nonadecane. It had a very small ion at *m/z* 198 (<1%) with additional ions at 180(1), 167(1), 149(1), 139(1), 134(2), 121(7), 111(5), 95(100), 93(25), 85(28), 79(10), 69(20), 68(25), 67(55), 55(33), 53(15), 43(20), and 41(35). Chemical ionization mass spectroscopy confirmed that the ion at 198 was the molecular ion.

Combined male head extracts were chromatographed on silicic acid with mixtures of pentane–methylene chloride–methanol. Elution with 10% CH_3OH in CH_2Cl_2 gave the 198 compound. From approximately 600 male *N. annulata* heads, 1 mg of the 198 compound was isolated. Its proton NMR spectrum showed: δ1.62,s,3H; 1.66,s,3H; 1.7,m,2H; 2.0–2.25,m,6H; 3.1,s,2H; 3.6,t(J = 7 Hz),2H; 4.14,d(J = 7 Hz),2H; 5.15,t(J = 7 Hz),1H and 5.4,t(J = 7 Hz),1H. The protons of δ3.1 exchanged with deuterium oxide. The ^{13}C spectrum gave additional information: δ62.38,t,(C-1); 124.49,d,(C-2); 138.68,s,(C-3); 29.89,t,(C-4); 25.64,t,(C-5); 123.85,d,(C-6); 135.07,s,(C-7); 29.68,t,(C-8), 36.15,t,(C-9); 59.06,t,(C-10); 15.74,q,(C-11); 15.77,q,(C-12).

The proton and [^{13}C]NMR data indicated two allylic methyl groups, a saturated CH_2, three allylic methylene groups, two hydroxyl groups, a methylene

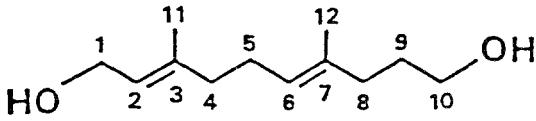

FIG. 1. Structure of 3,7-dimethyldeca-2,6-dien-1,10-diol.

group adjacent to oxygen, another methylene group both allylic and adjacent to an oxygen,and two vinyl hydrogens.

3,7-Dimethyldeca-2,6-dien-1,10-diol (Figure 1) seemed a suitable candidate. The proton NMR spectrum of our compound appeared compatible with the reported spectrum of synthetic diol prepared by Katzenellenbogen and Christy (1974) and more so with that reported by Masaki et al. (1985). The earlier reports of this compound did not have detailed proton NMR data (Meinwald et al., 1969; Miles et al., 1972; Masaki et al., 1980).

The diol was synthesized from all-*trans*-farnesol **2** (Figure 2) by the method of Hanzlik (1977) as developed by van Tamelen and Sharpless (1967) and van Tamelen et al. (1982). Attempts to selectively epoxidize the terminal double bond with *m*-chloroperbenzoic acid gave a mixture of epoxides at the terminal and central double bonds (Simon-Jordan, 1988). Similar attempts to prepare the diol using osmium tetroxide were unsuccessful. The epoxide **5** (obtained from bromohydrin **4**) was opened to the oxoacetate **6** with periodate (Boehm and Prestwich, 1986). Reduction of the oxoacetate **6** with lithium aluminum hydride gave the diol **1.** The synthetic scheme is shown below in Figure 2.

Behavioral Tests. The presence of 3,7-dimethyldeca-2,6-dien-1,10-diol at

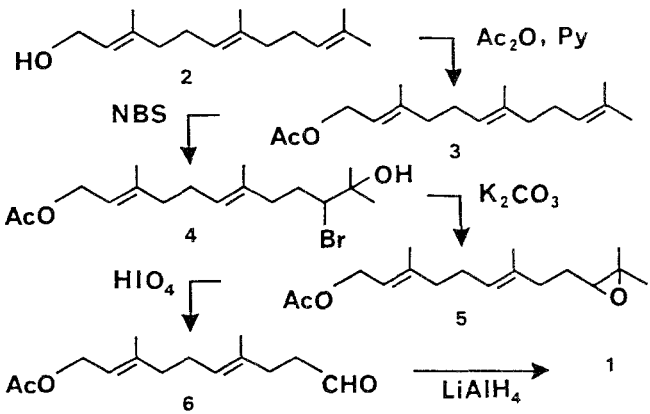

FIG. 2. Synthetic scheme for 3,7-dimethyldeca-2,6-dien-1,10-diol.

the *Andrena macra* nesting site did not cause any noticable change in the behavior of the male and female *Nomada annulata*. Responses for the three trials using standard diol were not significantly different from the control trials using a Mann-Whitney U test (Siegel, 1956). The trials employing male head extracts were also indistinguishable from the controls. Males were not attracted to the chemical source, nor were females. As individuals approached the chemical source, there was no noticeable increase in flight speed. *Nomada* did not land near the source. Neither males or females appeared to change direction or type of flight as they approached the chemical source.

DISCUSSION

3,7-Dimethyldeca-2,6-dien-1,10-diol (Figure 1) was first isolated from the danaid butterflies *Danaus gilippus berenice* and a subspecies *D. g. strigosus* along with an alkaloidal ketone (2,3-dihydro-7-methyl-1H-pyrrolizin-1-one) (Meinwald et al., 1969). While the ketone was shown to be a male pheromone (Schneider and Seibt, 1969), the only function attributed to the diol was that it imparted "the necessary stickiness to the carrier dust." It was suggested that the diol might have another function, mimicking the action of juvenile hormone (Pliske and Eisner, 1969). It is striking that diol **1** is present in males only of two such different insects as a butterfly and a *Nomada* bee. Its function remains to be determined in both cases.

Our results showed several important differences from those of Tengö and Bergström (1976, 1977). *Nomada annulata* release only a few micrograms of secretion per individual. In contrast, Tengö and Bergström reported that both sexes produce relatively large amounts of cephalic secretion (on the order of 1 mg/individual). Secondly, the major compound isolated from male *Nomada annulata* is not found in the Dufour's gland secretions of its host, *Andrena macra* (reported as *A. flexa* by Fernandes et al., 1981). The Dufour's glands of *A. macra* are dominated by farnesyl hexanoate and several other esters of farnesol. Of the seven *Nomada–Andrena* associations investigated by Tengö and Bergström, the male cephalic compounds of three do not match the major compound in the Dufour's gland of the host *Andrena* (Tengö and Bergström, 1977). The studies reported here add to the list of those showing differences. Obviously, there must be other factors involved in these *Nomada–Andrena* parasite–host associations. We raise the question as to whether chemical congruency between male nomadine cleptoparasites and the Dufour's gland secretions of their respective hosts is the norm or the exception. Certainly some *Nomada–Melitta* associations (Tengö and Bergström, 1976) show this chemical congruency, but such is not the case for *Holcopasites calliopsidis* and *Calliopsis andreniformis* (Hefetz et al., 1982). Unfortunately, we have so few chemical and biological

data on cleptoparasite–host associations that it is not yet possible to answer our question satisfactorily.

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CONIFERYL BENZOATE IN QUAKING ASPEN
A Ruffed Grouse Feeding Deterrent[1]

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Abstract—Quaking aspen (*Populus tremuloides* Michx.) staminate flower buds and catkins are important food resources for ruffed grouse (*Bonasa umbellus*); however, ruffed grouse select only certain quaking aspen to feed upon. Earlier studies indicate that the primary difference between quaking aspen that ruffed grouse feed upon and those not used is the level of coniferyl benzoate in the flower buds. Bioassays show that coniferyl benzoate is a feeding deterrent for ruffed grouse; its effect on ruffed grouse after ingestion has not been tested. Possible physiological effects, based on the chemical properties of coniferyl benzoate and its oxidation products, include inhibition of protein digestion, toxic effects, and antiestrogenic effects.

Key Words—Quaking aspen, *Populus tremuloides*, ruffed grouse, *Bonasa umbellus*, secondary metabolite, coniferyl benzoate, phenols, herbivore, plant defense, feeding deterrent.

INTRODUCTION

Ruffed grouse (*Bonasa umbellus*) rely on quaking aspen (*Populus tremuloides* Michx.) as a food source throughout the year, but are most dependent upon this species during winter and early spring (Gullion, 1966; Vanderschaegen, 1970; Svoboda and Gullion, 1972; Doerr et al., 1974; Huempfner, 1981). During many winters, the staminate flower bud of quaking aspen is consumed more frequently by ruffed grouse than any other food item (Vanderschaegen, 1970; Svoboda and Gullion, 1972; Doerr et al., 1974). Feeding on staminate flowers

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continues in the spring, when the buds expand to produce catkins. Ruffed grouse will feed almost exclusively on these catkins (Gullion, 1964; Vanderschaegen, 1970; Stoll et al., 1980).

Winter feeding observations indicate that ruffed grouse selectively feed in certain trees or clones of quaking aspen (Gullion, 1966; Huff, 1970; Schemnitz, 1970; Huempfner, 1981). It was reported (Jakubas et al., 1989) that the primary difference between quaking aspen that ruffed grouse fed upon and those not used as a food source was the level of coniferyl benzoate (CB) in their flower buds. Trees not used had significantly higher levels of CB in their flower buds. If CB is a feeding deterrent, it may influence the amount of quaking aspen that ruffed grouse consume in a given winter. Because quaking aspen is an important food for ruffed grouse, the welfare of a ruffed grouse population may be partially dependent on the availability of this food source (Gullion, 1969, 1977; Jakubas, 1989). Fluctuating levels of CB in quaking aspen may change its suitability as a food and affect ruffed grouse population levels by limiting the use of this important food resource (Jakubas, 1989).

Even though CB levels in quaking aspen flower buds and catkins are negatively related to ruffed grouse feeding preference, these correlations do not prove that CB acts as a feeding deterrent. To confirm that CB affects ruffed grouse feeding preference, bioassays must be conducted in which the feeding deterrent activity of coniferyl benzoate can be isolated from other factors that might influence ruffed grouse selection of aspen flower buds in the wild. This study was undertaken to confirm, through bioassays, that ruffed grouse feeding preference is affected by concentrations of CB normally found in quaking aspen flower buds.

METHODS AND MATERIALS

Synthesis and Extraction of Coniferyl Benzoate. Synthesis of CB was attempted using the method of Allen and Byers (1949) and by benzoylation of coniferyl alcohol (Hassner and Alexanian, 1978; Lindeberg, 1980). Intermediate and final products from each synthesis method were monitored by gas chromatography (GC), thin-layer chromatography (TLC), and high-pressure liquid chromatography (HPLC). Unknown products were identified by mass spectrometry. Mass spectral analyses were done on a Kratos MS25, with a Data General S/120 data system, using direct probe insertion. An HP 5890 GC with a J&W DB-5 column (30 m $\times$ 0.53 mm ID) was used for GC analyses. Methods for HPLC and TLC analyses are listed in Jakubas et al. (1989).

Mature quaking aspen with high concentrations of CB were felled for bud collection. Flower buds were stored on Dry Ice until they could be lyophilized. Lyophilized flower buds were ground in a Stein laboratory mill and extracted

in methylene chloride at room temperature for two days. The initial extract was decanted, and the buds were extracted a second time with methylene chloride overnight. The combined extracts were filtered and concentrated in vacuo using a Buchi rotary evaporator with a water bath at 30°C. Flash chromatography was used for initial purification of the concentrated extract (Still et al., 1978). The flash chromatography column was overloaded with extract and eluted with a 50:50 hexane–methylene chloride solution. A Waters 500 preparative HPLC system, with a single Waters Prepak silica gel cartridge was used to further purify the extract. A 40:60 hexane–methylene chloride solution was used as the mobile phase for preparative HPLC separations. Coniferyl benzoate content of the preparative HPLC fractions was monitored by TLC. HPLC fractions containing high levels of CB were evaporated using a rotary evaporator, with a water bath at 30°C. Final evaporation of any residual solvents was done under a stream of nitrogen. Coniferyl benzoate was crystallized by dissolving the extract in an ethyl ether–pentane solution and cooling overnight at −17°C (D. Joulain, Robertet, Grasse, France, personal communication).

Coniferyl benzoate crystals were dissolved in ethyl ether and quantified by HPLC. Pure CB (courtesy of D. Joulain) and an internal standard of pinosylvin dimethyl ether (courtesy of T. Clausen, University of Alaska) were used to create a calibration curve. Analytical HPLC conditions follow those in Jakubas et al. (1989). Because of the large amount of CB needed for the bioassays (approx. 20 g), additional CB was recovered by adding CB of lower purity (approx. 70–80%) to the ether solution containing crystalline CB. Coniferyl benzoate levels in the final solution were quantified by HPLC.

Extraction of CB from aspen flower buds using this procedure was very time consuming. Initial purification of the extract by liquid–liquid partitioning followed by flash chromatography and crystallization should be more time and cost efficient. The concentration of CB in gum benzoin Siam is much higher than that found in quaking aspen flower buds and is a better natural source of CB (Freudenberg and Bittern, 1950; D. Joulain, personal communication). However, not all samples of raw gum benzoin Siam contain CB.

Preparation of Bioassay Diets. Freudenberg and Bittern (1950) reported that CB is sensitive to heat, oxygen, acids, and alkalies; therefore, the stability of CB had to be tested before the bioassays could be conducted. Pure CB was dissolved in ether and applied in equal quantities to both turkey chow and glass beads. Following evaporation of the ether solution, the glass beads and turkey chow were stored in open containers at −17°C, 23°C, and 110°C for 24 hr. These samples were compared by TLC to a control that consisted of treated glass beads stored under nitrogen at −17°C. Only the samples that were stored at 110°C showed signs of degradation after 24 hr. Therefore, degradation of CB during the daily feeding trials was not expected.

Coniferyl benzoate was added to game bird feed by mixing an ether solution containing a known amount of CB with Glenco (Glenco Mills, Division of International Multifoods, Inc.) pelleted game bird feed. The ether was evaporated from the feed using a rotary evaporator with a water bath at 30°C, followed by drying under nitrogen.

Bioassays. Past attempts to keep wild adult ruffed grouse in captivity have proven to be difficult; therefore, ruffed grouse were raised from eggs obtained from wild birds (courtesy of F. Thompson, University of Missouri) or from local captive birds. Ruffed grouse were kept in outdoor cages and conditioned to a standard diet of game bird feed for one month prior to the bioassays. During this time, ruffed grouse were fed ad libitum, and their daily food consumption was monitored.

All bioassays consisted of presenting captive ruffed grouse a paired choice of treatment and control diets. Diets were randomly placed in identical feeding trays. To prevent the birds from associating tray location with a particular diet, the order of the trays was switched when a feeding trial was repeated. Feeding trials were repeated a second day except in bioassay 2. Each treatment and control diet contained a quantity of feed equivalent to the normal average daily consumption of an individual bird. Bioassays were conducted in December 1988 at the Minnesota Department of Natural Resources' Carlos Avery Game Farm.

Six ruffed grouse were originally used for the bioassays (except for bioassay 2). One bird showed irregular feed consumption before and during the feeding trials and died two days after all the bioassays were completed. Death was apparently due to kidney failure (renal tubular nephrosis) (D. Shaw, University of Minnesota Veterinary Diagnostic Laboratories). All bioassay data from this bird were omitted. It is not believed that the bioassays caused the nephrosis.

Statistical Analyses. Results from the bioassays were analyzed using paired t tests. When a feeding trial was repeated, the amounts of the treatment and control diets consumed were respectively combined and analyzed. Results were considered significant when $P < 0.05$.

RESULTS

Coniferyl Benzoate Synthesis. Synthesis of CB using the method of Allen and Byers (1949) was unsuccessful. Although coniferyl alcohol was made by this method, the benzoylation procedure did not produce CB but rather two products closely related to CB. These products were identified by mass spectrometry as coniferyl dibenzoate and an isomer of CB [4-(3-hydroxy-1-propenyl)-2-methoxyphenyl benzoate] (Figure 1). Likewise, benzoylation of coniferyl alcohol, following the room temperature esterification method of Hassner and Alexanian (1978), resulted in the same two by-products plus a

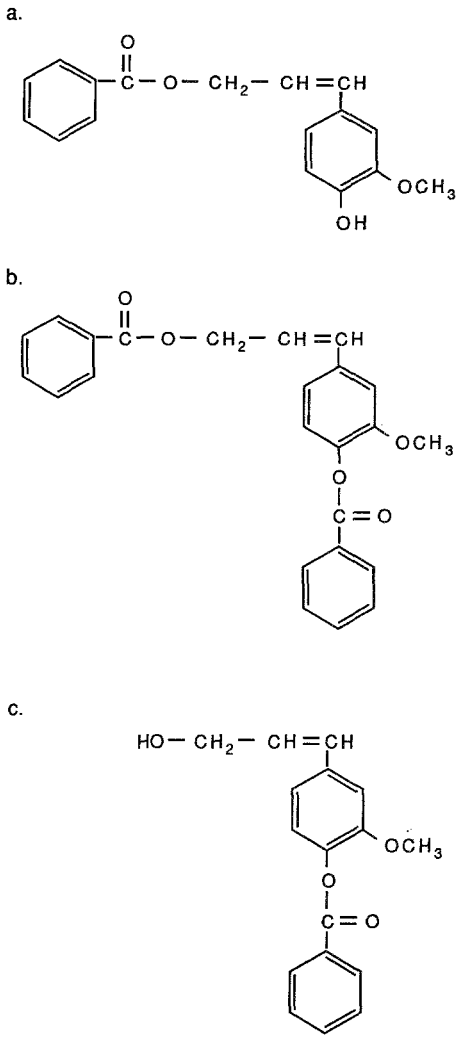

FIG. 1. Major products of coniferyl benzoate synthesis: (a) coniferyl benzoate, (b) coniferyl dibenzoate, and (c) 4-(3-hydroxy-1-propenyl)-2-methoxyphenyl benzoate.

minor amount of CB. Because of the lability of CB, protecting the phenol group of coniferyl alcohol prior to benzoylation would be difficult (G. Gray, University of Minnesota, personal communication). Cleavage of coniferyl dibenzoate to CB using a 1% (w/v) solution of potassium carbonate in methanol or a 0.05 N solution of sodium hydroxide in methanol was also unsuccessful. The main

TABLE 1. QUANTITY OF CONTROL (C) AND TREATMENT (T) DIETS PRESENTED AND CONSUMED FOR EACH BIOASSAY, AND STATISTICAL SIGNIFICANCE AS DETERMINED BY PAIRED *t* TESTS

	Total weight (g)									
	Amount presented		Amount consumed		Mean consumption (g)/day/bird ± SE		% of total eaten			
Bioassay	T	C	T	C	T	C	T	C	*df*	Level of significance (*P*)
1[a]	446	446	153	377	15.3 ± 3.7	37.7 ± 2.8	29	71	4	0.005
2[b]	164	164	54	132	13.4 ± 5.5	33.0 ± 7.6	29	71	3	0.11
3[c]	500	500	216	299	21.6 ± 5.1	30.0 ± 4.1	42	58	4	0.23

[a] Control and treatment diets consisted of commercial feed treated with ether and 2.85% (dry wt) coniferyl benzoate, respectively; bioassay replicated a second day.
[b] Control and treatment diets consisted of commercial feed treated with 0.99% and 2.85% (dry wt) coniferyl benzoate, respectively; bioassay not replicated.
[c] Control and treatment diets consisted of commercial feed treated with ether and 0.42% (dry wt) solution impurities; bioassay replicated a second day.

products of the cleavage were the isomer of CB and an unknown compound, which likely resulted from the cleavage of both benzoate groups.

Bioassay 1. This bioassay tested the ability of ruffed grouse to differentiate between feed treated with CB and untreated feed. Ruffed grouse were presented feed treated with CB, at a concentration found in unused quaking aspen[3] (2.85% dry wt), and a control diet consisting of feed treated with ether. Ruffed grouse ate significantly more of the ether control diet than feed containing CB (Table 1). Ruffed grouse uniformly preferred the control diet over feed treated with CB during the two days this trial was conducted.

Bioassay 2. This bioassay tested the birds' ability to discriminate between levels of CB found in preferred[4] and unused quaking aspen. Ruffed grouse were presented a choice between feed containing 2.85% or 0.99% (dry wt) CB, which correspond to levels found in preferred and unused quaking aspen respectively. Because of an insufficient quantity of CB, this trial was conducted with only five birds (one of which was later omitted as explained above) and could not be repeated.

[3] Unused quaking aspen buds collected during 1985 and 1986 had a mean concentration of CB of 2.28% (dry wt), with concentrations ranging from 0.66 to 3.68% (dry wt). Quaking aspen were classified as unused trees if ruffed grouse had never been observed feeding in them.
[4] Preferred quaking aspen buds collected during 1985 and 1986 had a mean concentration of CB of 0.99%, with concentrations ranging from 0.52 to 1.57% (dry wt). Quaking aspen were classified as preferred trees if ruffed grouse were currently feeding on them.

Three of four ruffed grouse ate more of the feed containing 0.99% CB than feed containing 2.85% CB. However, one bird ate essentially equal amounts of both diets, making the overall difference in consumption between the two diets statistically insignificant (Table 1). Preference for the diet containing less CB was clear among the birds that did discriminate between the two diets. These birds collectively consumed 3.3 times more of the diet containing 0.99% CB than the 2.85% diet.

Bioassay 3. Because of the minor impurities in the extracted CB, an additional trial was conducted to determine if the impurities were affecting ruffed grouse food choice. The extracted CB was estimated to be 87% pure (by HPLC–diode array detection). Chromatography fractions that contained the same spectrum of impurities found in the extracted CB were combined and concentrated. The concentrated "impurities" were weighed and added to the game bird feed using the same methods that were used to apply CB. Ruffed grouse were presented feed that contained 0.42% (dry wt) "impurities" and a control diet consisting of feed treated only with ether.

Ruffed grouse did not discriminate between feed treated with "impurities" and the ether control diet over the two day testing period (Table 1). Combining the individual feeding trial results for statistical analyses may have overestimated (based on the size of the P value) the birds' ability to discriminate between the two diets. Feed treated with "impurities" on the first day of this trial still retained a faint odor of ether. By the second day this odor had disappeared, and the birds showed less discrimination between the two diets. Significance levels for the individual trials were $P = 0.21$ and $P = 0.91$ for the first and second day, respectively.

DISCUSSION

Collectively, these bioassays support field observations which indicate that the concentration of CB in quaking aspen flower buds is an important factor mediating ruffed grouse food selection. Coniferyl benzoate, when applied to commercial feed at concentrations found in unused aspen flower buds, acts as a feeding deterrent.

The first bioassay, a feeding trial which demonstrated that ruffed grouse will select a standard commercial diet over a diet containing CB, does not in itself verify that CB is a feeding deterrent. Ruffed grouse preference for the standard diet may have been due simply to the birds avoiding feed treated with a novel substance. To control for this novelty effect, ruffed grouse were presented with two diets containing different levels of CB (bioassay 2). This bioassay also tested if ruffed grouse could discriminate between different CB levels commonly found in aspen flower buds. The majority of ruffed grouse showed

a preference for the diet that had the lower level of CB. However, one bird ate nearly equal amounts of both diets making the test statistically insignificant. The small number of ruffed grouse used in this trial might have contributed to the low level of statistical significance. These statistics do not reflect the order in which the feed was consumed. All of the birds that were able to discriminate between the two diets consumed all of the feed containing low levels of CB first, and then proceeded to eat the feed treated with higher levels of CB. This lowered the difference in consumption between the two diets and the level of significance for the bioassay. Finally, animals vary in their ability to perceive chemical stimuli, and one should not expect that all ruffed grouse have the same ability to detect CB. The overall response of ruffed grouse to the second bioassay, and the results of the third bioassay, in which the birds did not discriminate between the standard diet and feed treated with a novel substance (the CB impurities in this case), indicate that ruffed grouse were not discriminating between diets simply because one diet was treated with a novel substance, but rather were deterred by the concentration of CB in the diet.

The variance in the ability of ruffed grouse to detect CB may be an important factor in determining how well individual ruffed grouse adapt when their natural diet shifts to a higher percentage of aspen flower buds in the fall and winter. Ruffed grouse may detect CB in aspen flower buds through their gustatory or trigeminal sensory systems. Preliminary data indicate that visual factors such as aspen bud color or the amount of external resin on the bud are not important in the selection of aspen flower buds (Jakubas, 1989). Rather, a burning sensation (human perception), which occurred near the soft palate and in the throat when tasting aspen flower buds, was found to be significantly related to the level of CB in the buds (Jakubas, 1989). Taste preferences are often poorly correlated between species (Arnold and Hill, 1972; Oates et al., 1980). However, the burning sensation experienced from ingesting CB was not a true taste perception but likely was associated with the trigeminal sensory system. Trigeminal chemoreception is often referred to as the "common chemical sense" in vertebrates, in part, because of the unspecialized nature of the chemical receptors (i.e., free nerve endings) (Kare and Mason, 1986; Silver, 1987). Therefore, the burning sensation produced by coniferyl benzoate could be perceived by ruffed grouse (Jakubas, 1989). The feeding deterrent property of CB does not appear to be dependent on ruffed grouse learning to associate CB with some type of gastrointestinal disturbance. During the bioassays ruffed grouse were observed to avoid feed treated with CB after only a few of the treated pellets were consumed, which would have been too soon for the birds to have experienced any adverse dietary effects.

The effect that CB has on ruffed grouse once it is ingested may be dependent on the fate of coniferyl alcohol in the digestive system of the bird. Coniferyl alcohol could result from cleavage of CB by acid hydrolysis during

digestion. Since CB appears to be a good alkylating agent, the coniferyl alcohol radical could bind to nucleophilic sites on dietary proteins and affect protein digestion (Jakubas et al., 1989). If CB does bind to dietary proteins, the reduction in the amount of protein ruffed grouse could obtain from aspen flower buds could be significant. Aspen flower buds typically contain about 10% crude protein. If a ruffed grouse were to feed on aspen flower buds that have a CB concentration between 2% and 4% (dry wt), which is typical of flower buds from unused trees, its intake of CB could be sufficient to lower the amount of digestible protein past the point where it is profitable for the bird to feed on that tree. Andreev (1988) reported that the total digestibility of the natural foods of other grouse species depends on the amount of protein in their diet. A loss of dietary protein could ultimately affect a bird's energetics, reproduction, and ability to escape predation.

The high reactivity of CB makes speculation on its fate in the bird's digestive system difficult at best. Instead of coniferyl alcohol binding to nucleophilic protein sites, it might polymerize, as it does when it forms lignin in plants, and have little effect on the bird. Alternatively, coniferyl alcohol has been shown to have strong antifungal properties (Keen and Littlefield, 1979; Hammerschmidt and Kuc, 1982; Kuc, 1983). The antifungal property of coniferyl alcohol may affect the microflora in the ceca and intestines of the bird, thereby affecting digestion and detoxification processes. If coniferyl alcohol were to oxidize, the oxidation products could be toxic to the bird or have antiestrogenic effects. Ferulic acid, which would result from oxidation of the primary alcohol of coniferyl alcohol, has been shown to have antiestrogenic properties in *Microtus montanus* and Japanese quail (*Coturnix coturnix*) (Berger et al., 1977; DeMan and Peeke, 1982).

The biological effects of CB should not be thought of as being due entirely to coniferyl alcohol. Hjorth (1961), when investigating the skin irritant properties of CB, found that applications of CB resulted in stronger and more frequent allergic responses than coniferyl alcohol. The specific chemical properties of the coniferyl alcohol ester (CB) appear to increase its biological activity as compared to the alcohol. Certainly, determining the physiological effects of CB on ruffed grouse will be valuable in understanding how this compound affects the overall ecology of the bird.

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DIVERSITY IN DIGESTIVE PROTEINASE ACTIVITY AMONG INSECTS

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Abstract—pH-optimum curves for proteolytic activity from midgut homogenates of 23 species of insects were determined using a radiometric proteinase assay. Optima for digestion of radiolabeled methemoglobin in these insects ranged from pH 3.0 to pH 12.0. Since pH optima vary among the different mechanistic classes of proteinases, these data suggest that there is high diversity in midgut digestive proteinases among various insects.

Key Words—insect digestion, insect digestive proteinases, insect midgut, pH optimum.

INTRODUCTION

The digestive proteinases of insects catalyze the release of free amino acids from dietary protein and thereby provide a supply of nutrients essential for normal growth and development. There are four mechanistic classes of proteinases: aspartic (also called carboxyl or acid), cysteine (also called thiol), serine, and metalloproteinases (Barrett, 1986). All insect digestive proteinases studied to date belong to the first three classes (Applebaum, 1985). For many years most insects were presumed to utilize serine proteinases with trypsin or chymotrypsin-like specificities to digest their dietary protein although recent work has shown this not to be the case (Murdock et al., 1987).

Information about insect digestive proteinases is necessary to understand insect growth and development, processes that depend upon these enzymes. Digestive proteinases in herbivorous species are also the purported target of plant proteinase inhibitors: they are the sites of biochemical vulnerability through which these potentially important plant allelochemicals may impact insect

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growth and development. Individual proteinase inhibitors have a limited spectrum of activity and often are active against only one class of proteinases. Therefore, the potential for an inhibitor to be active against a specific insect would depend upon the proteinase type(s) present in the insect's midgut.

The names of the classes of proteinases reflect the key catalytic moiety (an amino acid or cation) participating in the cleavage of the peptide bonds. In practice, the mechanistic class to which a proteinase belongs is first inferred from its in vitro characteristics, including (1) the pH range over which it is maximally active; (2) its sensitivity to various inhibitors; (3) its ability to hydrolyze specific proteins or peptides; and (4) its similarity to well-characterized proteinases (North, 1982; Barrett, 1986; Wagner, 1986). In brief, some characteristics of digestive proteinases utilized by insects are as follows: serine proteinases (e.g., trypsin and chymotrypsin) are generally active in the pH 7.0–10.0 range and are inhibited by such well-known inhibitors as Bowman-Birk, Kunitz, and lima bean inhibitor; cysteine proteinases (e.g., papain) are generally most active in the mildly acid range, pH 5.0–7.0, are inhibited by heavy metals, cystatin, and E-64, and are enhanced by reducing agents; aspartic proteinases (e.g., pepsin) are active in the acid pH range, generally below pH 4.5, and are inhibited by pepstatin (Barrett, 1977; North, 1982).

In the present paper we report on some characteristics of the proteolytic activity observed in midgut homogenates of a variety of insect species. Our earlier studies suggested that digestive cysteine proteinases are more widely distributed in insects than had been previously assumed (Murdock et al., 1987). Accordingly, we undertook a survey of a wider range of insect species to gain a better understanding of the diversity among proteinases in insects. We used as our primary indicator the characteristic relationship between proteolytic activity and pH. We were not selective in our choice of insects; rather, we used those species we were able to obtain or that could be collected in the area of Lafayette, Indiana.

METHODS AND MATERIALS

Our sample of insects included two lepidopterous larvae: cabbage looper [*Trichoplusia ni* (Hubner)] and green cloverworm [*Plathypena scabra* (Fabricius)], both Noctuidae; nine species of Coleoptera: larval Mexican bean beetle [*Epilachna varivestis* Mulsant], a coccinellid; larval three-lined potato beetle [*Lema trilineata* (Olivier)], larval asparagus beetle [*Crioceris asparagi* (L.)], adult and larval western corn rootworm [*Diabrotica virgifera virgifera* LeConte], southern corn rootworm larvae (from diet) and adults (from the field) [*Diabrotica undecimpunctata howardi* Barber], and soybean leaf miner adults [*Odontota horni* (Smith)], all chrysomelids; margined blister beetle [*Epicauta*

pestifera Werner], family Meloidae; boll weevil (from diet) [*Anthonomus grandis grandis* Boheman], family Curculionidae; *Caryedes braziliensis* Thunberg, family Bruchidae; two Hymenoptera: the grass sawfly [*Pachynematus extensicornis* (Norton)], family Tenthredinidae, and the European pine sawfly [*Neodiprion sertifer* (Geoffroy)], family Diprionidae; one species of Neuroptera: a hellgrammite [*Corydalus cornutus* (L.)], family Corydalidae; one species of Ephemeroptera: mayfly nymphs [*Ephoron* sp.], family Polymitarcyidae; one species of Trichoptera: caddisfly larvae [*Macrostemum zebratum* (Hagen)], family Hydropsychidae; four hemipterans: green stink bug adults [*Acrosternum hilare* (Say)], family Pentatomidae; a mirid [*Miris dolobratus* (L.)], family Miridae; adult coreid bugs collected from jimsonweed (species unknown) and squash bug nymphs [*Anasa tristis* (De Geer)], both family Coreidae; one homopteran: a leafhopper, family Cicadellidae (species unknown); one dipteran: crane fly larvae, family Tipulidae (species unknown); and one odonate: adult dragonflies [*Libellula luctuosa* Burmeister]. (Species identification was provided by the collector of the specimens, most frequently Mr. Dan Bloodgood for aquatic insects, and Mr. Robert Meyer for the terrestrial species.)

The midguts of all insects were dissected out as soon as specimens were brought into the laboratory. Only insects with full guts were used. Most herbivorous insects were taken directly off their host plants, although some had been reared on artificial diet (these are noted in the species listing). A typical sample consisted of pooled midguts from at least 10 individuals. Upon dissection, the midguts were placed immediately into cold, ultrafiltered water. The midguts were then homogenized with a glass homogenizer and centrifuged at 12,800*g* for 5 min. The supernatant was divided into aliquots that were frozen at −80°C until assayed. If the size of the midguts necessitated homogenization of subsamples, the supernatants of the subsamples were pooled, mixed, and divided into aliquots prior to freezing. Proteinase activity in each midgut preparation was diluted to be within the linear range of the assay.

Proteinase Assay. To measure proteolytic activity, we used a highly sensitive proteinase assay used earlier in our laboratory (see Shukle et al., 1985; Kitch and Murdock, 1986, for method details). In brief, the method consisted of incubating tritiated protein substrate (methemoglobin in these assays) with insect midgut homogenates at 37°C for 10 min. As a result of proteolytic activity, the radioactive substrate is cut, releasing small radioactive peptides. Unlike the parent protein, these small radioactive peptides are soluble in 5% trichloroacetic acid and can be quantitatively determined by liquid scintillation counting of the supernatant after centrifugation. Characteristics of the proteinases in the midgut can be assessed by comparing the quantity of TCA-soluble radioactivity released under different assay conditions, e.g., at different pHs. This assay requires only miniscule amounts of insect gut homogenate per assay (less than one one-hundredth of a gut equivalent per assay is not unusual),

and, unlike many other proteinase assays, it measures the ability of the proteinase to digest a protein, not its esterolytic activity on a synthetic substrate.

The following buffers were used in pH activity determination assays: pH 2.0–3.0, 200 mM glycine HCl; pH 3.5–4.5, 200 mM beta-alanine HCl; pH 5.0–5.5, 200 mM sodium acetate; pH 6.0–7.5, 100 mM sodium phosphate biphosphate; pH 8.0–8.5, 100 mM Tris HCl; pH 9.0–10.5, 200 mM glycine NaOH; and pH 11.0–12.0, 100 mM sodium phosphate NaOH. All buffers were adjusted to the conductivity of 150 mM NaCl. pH optima were determined in the presence of 5 mM cysteine, a reducing agent that enhances activity of cysteine proteinases. Activity over the pH range tested was expressed as percent of the maximum activity measured.

The inhibitors used and the final concentrations at which their activity was assessed were as follows: E-64 (cysteine proteinase inhibitor), 100 μg/ml (280 μM); leupeptin (an inhibitor of both serine and cysteine proteinases), 10 μg/ml (21 μM); pepstatin (an aspartic proteinase inhibitor) 100 μg/ml (145 μM); *p*-hydroxymercuribenzoic acid (pHMB) (which inhibits cysteine proteinases), 0.1 mM; Kunitz (a serine proteinase inhibitor), 100 μg/ml; lima bean (a serine proteinase inhibitor), 100 μg/ml; antipain (an inhibitor of aspartic and serine proteinases), 10 μg/ml (all inhibitors were purchased from Sigma Chemical Co.). Inhibitory activity was assessed at the pH value for which a midgut preparation demonstrated maximum activity. Inhibitors were preincubated with the enzyme preparation for at least 10 min prior to adding radioactive substrate to begin the reaction. All assays were run in duplicate. The activity of the substrate in the absence of the proteinase preparation was assessed at each pH and this control value substracted from the proteolytic activity observed at each pH.

RESULTS AND DISCUSSION

The pH optimum curves for the 23 species examined are presented in Figure 1. While our sample is too small to make statements about insects in general, our data do suggest that there is a tremendous diversity in digestive proteinase activity among insect species. Insects with proteolytic activity in the acid to mildly acid range are quite common; 14 of the 23 insects examined in this study exhibited major peaks of activity at pH 6 or below. The pH profiles for some insect midgut homogenates exhibit multiple peaks suggesting that these insects might utilize multiple enzymes.

There are species that exhibit proteolytic activity at distinctly acid pH values (e.g., the leafhopper, blister beetle; Figures 1M and W), others where maximal activity is in the very basic range (e.g., green cloverworm, grass sawfly larvae, crane fly larva; Figures 1B, O, X), while others have most of their proteolytic activity in the mildly acidic range (e.g., southern corn rootworm

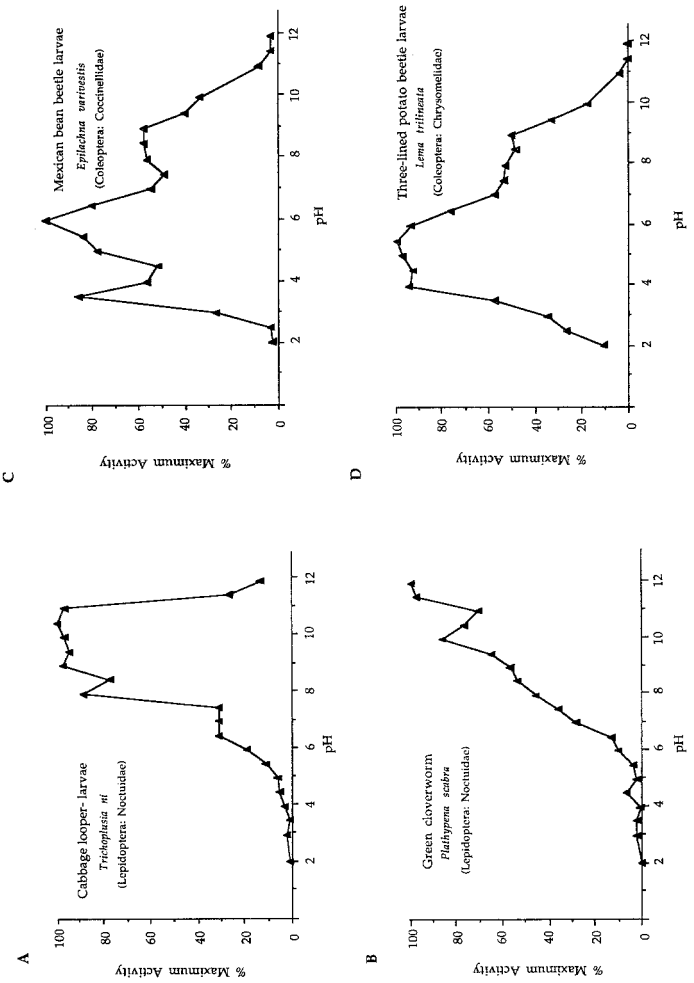
adults, green stinkbug; Figures 1J and S), mildly basic (e.g., southern corn rootworm larvae, *Caryedes brazilansis*; Figures 1I and L), or even in a broad range from acidic to basic pH (e.g., Mexican bean beetle, hellgrammite, mayfly nymph; Figures 1C, N, and R). Our data indicate no obvious relationship between the feeding habit of the insect and the pH optimum for proteolytic activity. The pH optimum for herbivorous insects ranged from pH 3.0 to pH 12.0. The lepidoptera examined demonstrate serine-like activity, as would be expected from the literature. Coleoptera show a high diversity even within a family; the chysomelids exhibit pH optima from 4–5 (soybean leaf miner, Figure 1F) to pH 9–11 (Southern corn rootworm-larvae, Figure 1I).

The sensitivity of selected enzymes to a series of inhibitors and the pH at which they were tested are presented in Table 1. In general, the susceptibility to inhibition exhibited by the midgut homogenates generally confirms the presence of the type of proteinase suggested by the pH optimum curves. The sensitivity of the midgut proteolytic activity to the inhibitors was tested at the pH optimum for proteolytic activity. Therefore, in many instances, the pH at which the assay was conducted was far from the pH at which the inhibitor would be expected to be active (e.g., testing Kunitz inhibitor, a serine proteinase inhibitor, at pH 3). For these reasons the results of the inhibitor assays should be interpreted with caution.

Although the assay we used only provides information on activity over the pH range tested, pH optimal activity is a useful characteristic (although not the diagnostic characteristic) of different classes of proteinases. Insects in which pH optima have been assessed using this assay [*Callosobruchus maculatus* F., *Mayetiola destructor* (Say) and *Acanthoscelides obtectus* Say] have been found, on further purification, to contain the proteinase type suggested by the radiometric assay (Shukle et al., 1985; Kitch and Murdock, 1986; Wieman and Nielsen, 1988). Additionally, results of insect bioassays have indicated that ingestion of proteinase inhibitors active against the dominant enzyme class suggested by the radiometric assay can have a severe impact on insect growth and development (Wolfson and Murdock, 1987; Murdock et al., 1988). In contrast, even when tested at similar or higher concentrations, potentially active inhibitors of proteinases not apparently predominant in the midgut have little or no impact on growth and development (Wolfson, 1990).

Midgut homogenates are crude mixtures of enzymes and food substrate. Although we cannot comment on possible proteolytic activity in the midgut homogenates of predatory or aquatic insects, we have performed proteinase assays using leaf extracts from a substantial number of plant species and these indicate that proteolytic activity of plant origin would, at most, make a very small contribution to the total proteolytic activity found in insect midgut homogenates (Wolfson and Murdock, unpublished).

The diversity in proteolytic activity among insects should be kept in mind


A
Cabbage looper- larvae
Trichoplusia ni
(Lepidoptera: Noctuidae)
% Maximum Activity
pH
B
Green cloverworm
Plathypena scabra
(Lepidoptera: Noctuidae)
% Maximum Activity
pH
C
Mexican bean beetle larvae
Epilachna varivestis
(Coleoptera: Coccinellidae)
% Maximum Activity
pH
D
Three-lined potato beetle larvae
Lema trilineata
(Coleoptera: Chrysomelidae)
% Maximum Activity
pH

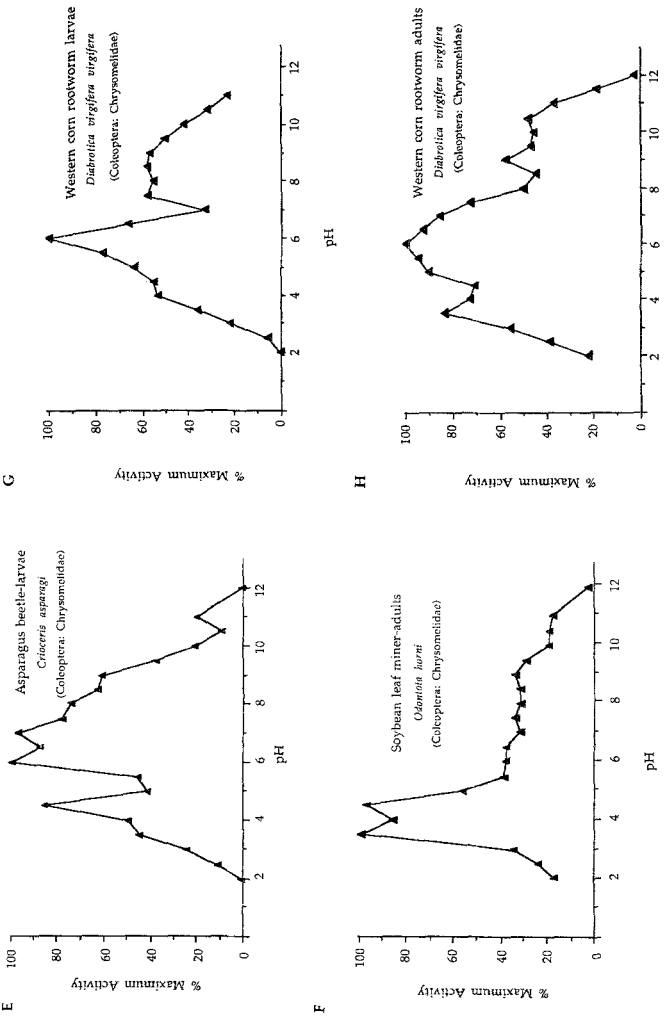
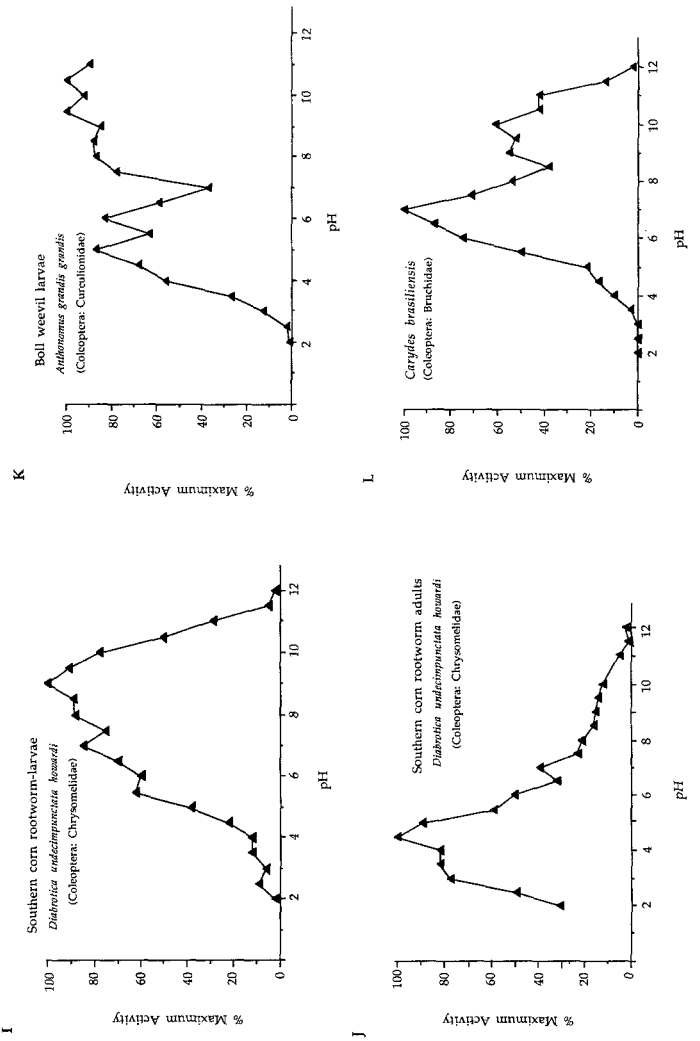


FIG. 1. pH optima of proteolytic activity (as measured by methemoglobin digestion) of midgut homogenates from various insects.


I
Southern corn rootworm-larvae
Diabrotica undecimpunctata howardi
(Coleoptera: Chrysomelidae)
% Maximum Activity
pH
J
Southern corn rootworm adults
Diabrotica undecimpunctata howardi
(Coleoptera: Chrysomelidae)
% Maximum Activity
pH
K
Boll weevil larvae
Anthonomus grandis grandis
(Coleoptera: Curculionidae)
% Maximum Activity
pH
L
Carydes brasiliensis
(Coleoptera: Brachidae)
% Maximum Activity
pH

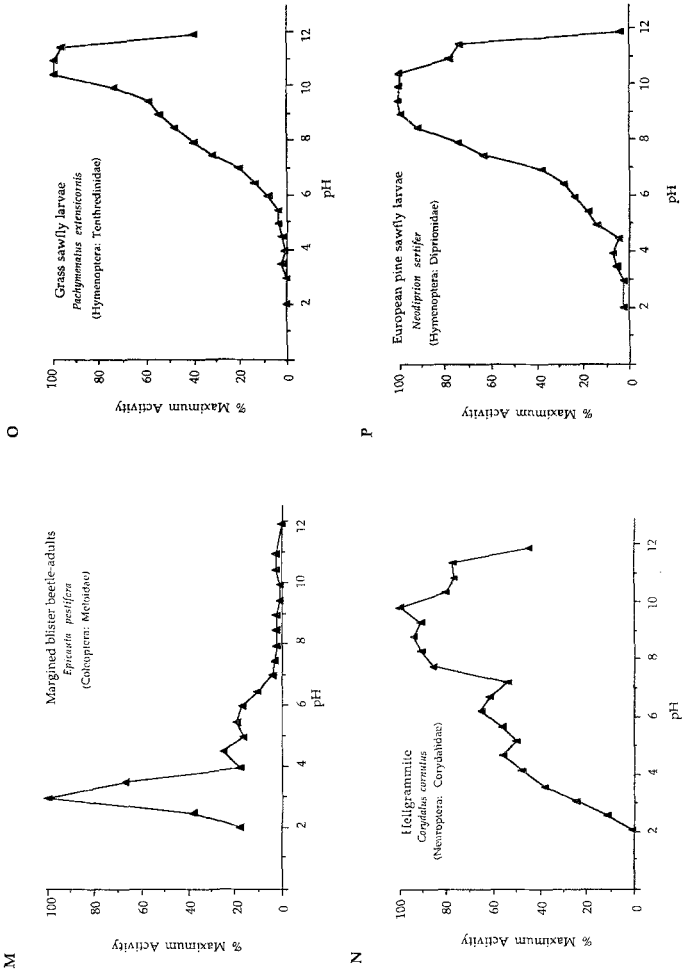
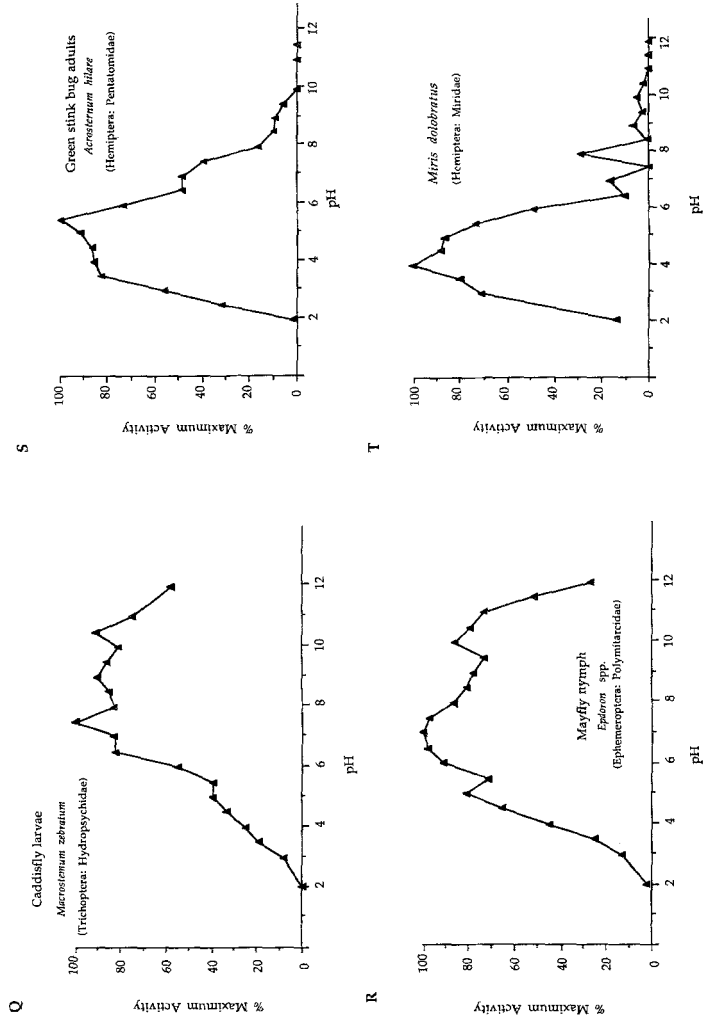


FIG. 1. Continued.


Q
Caddisfly larvae
Macrostemum zebratum
(Trichoptera: Hydropsychidae)
% Maximum Activity
pH
R
Mayfly nymph
Ephoron spp.
(Ephemeroptera: Polymitarcidae)
% Maximum Activity
pH
S
Green stink bug adults
Acrosternum hilare
(Hemiptera: Pentatomidae)
% Maximum Activity
pH
T
Miris dolobratus
(Hemiptera: Miridae)
% Maximum Activity
pH

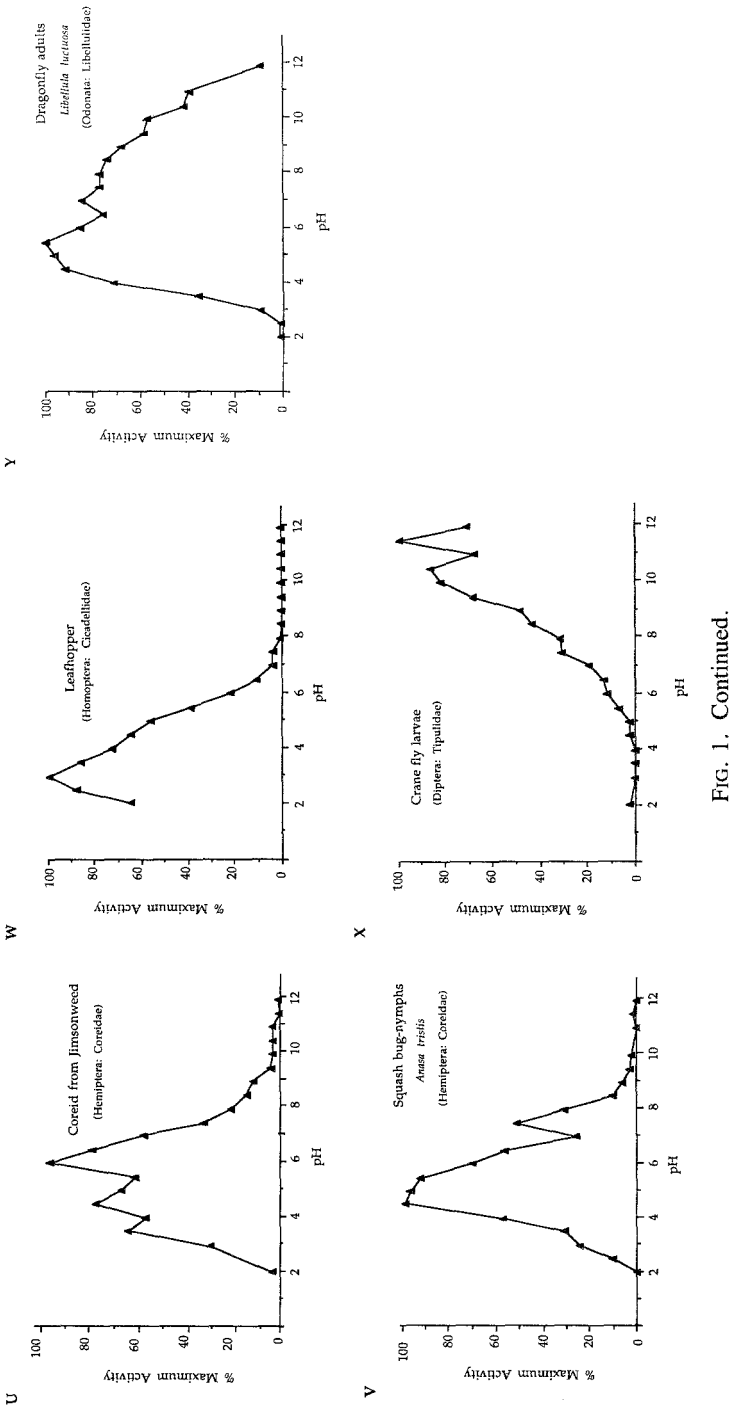


FIG. 1. Continued.

TABLE 1. PERCENT INHIBITION (−) OR STIMULATION (+) OF MIDGUT PROTEOLYTIC ACTIVITY WHEN MIDGUT HOMOGENATE IS PREINCUBATED WITH PROTEINASE INHIBITORS (SPECIFICITIES IN PARENTHESES): E-64 (CYSTEINE), LEUPEPTIN (SERINE, CYSTEINE), PEPSTATIN (ASPARTIC), pHMB (CYSTEINE), KUNITZ (SERINE), LIMA BEAN (SERINE), AND ANTIPAIN (SERINE, ACID)[a]

	Inhibitors and enhancers										
Insect species	pH	E-64	leupep.	pepstat.	pHMB	L-cyst.	L-cyst. + pHMB	Kun.	L.B.	antip.	Possible proteinase
Leafhopper	3.0	−1	−64	−95	−16	+10	+1	−3	−3	−2	acid
Epicauta pestifera	3.0	−88	−78	−42	−88	+155	+211	−25	−33	−91	acid, cysteine
Miris dolobratus	3.0	−37	−85	−63	+18	+50	+59	−30	−58	−30	acid, cysteine
Diabrotica undecimpuncata (adult)	4.5	−7	−2	−79	−14	+14	−3	−83	−84	−88	acid
Odontota horni	4.5	−52	−51	−59	−75	+56	−17	−72	−75	−97	acid, cysteine
Anasa tristis	4.5	−10	−9	−32	−51	+77	+101	+12	+5	−14	acid, cysteine
Lema trilineata	5.5	−95	−91	−26	−66	+43	+48	−67	−56	−26	acid, cysteine, serine
Acrosternum hilare	5.5	−81	−66	−5	−84	+85	+103	−46	−32	−71	cysteine, serine
Libellula luctnosa	5.5	−10	−44	−35	−2	+7	−4	−98	−88	−50	acid, serine
Epilachna varivestis	6.0	−63	−100	−26	−75	+199	+179	−8	−8	−58	acid, cysteine
Crioceris asparagi	6.0	−92	−73	+7	−85	+147	+250	−10	−25	−94	cysteine, serine
Jimson correid	6.0	−94	−91	+5	−96	+62	+60	−40	−58	−95	cysteine, serine
Euphoron spp.	7.0	−25	−54	−24	−5	+5	+1	−99	−95	−58	serine
Macrostemum zebratum	7.5	−44	−62	−10	+1	+9	+3	−99	−87	−72	cysteine, serine
Corydalus cornutus	9.0	−19	−26	−12	−3	+9	+4	−94	−74	−32	serine
Trichoplusia ni	10.0	−46	−63	+10	−54	+12	−7	−91	−86	−79	serine
Plathypena scabra	10.0	−13	−20	−12	−22	+6	+9	−81	−65	−40	serine
Neodiprion sertifer	10.0	−4	−70	+9	+3	+15	+20	−98	−86	−74	serine
Pachynematus extensicornis	11.0	−24	−61	−15	+8	+21	+10	−92	−83	−55	serine
Crane fly sp.	11.0	−11	−12	−15	−38	−14	−29	−100	−97	−42	serine

[a] L-Cysteine enhances cysteine proteinases and reverses inhibition by pHMB.

when evaluating the potential importance of proteinase inhibitors as allelochemicals active against leaf feeding insects. Information on the digestive proteolytic activity in a given insect species should be available before the possible importance of proteinase inhibitors, either induced or constitutive, is invoked to describe aberrant growth patterns. Proteinase inhibitors could be responsible for certain growth patterns observed with some plant–insect combinations, but evidence supporting the susceptibility of the insect's digestive enzyme to the inhibitor should be presented before making assertions about such cause-and-effect relationships.

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MANDELONITRILE IN LARVAL SECRETION OF MOUNTAIN ASH SAWFLY, *Pristiphora geniculata* (HYMENOPTERA: TENTHREDINIDAE)

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Abstract—The larval defensive secretion of the mountain ash sawfly, *Pristiphora geniculata*, contains seven volatile components. They include benzaldehyde, borneol, bornyl acetate, mandelonitrile, and C_{14}, C_{16}, and C_{18} acetates.

Key Words—Mountain ash sawfly, *Pristiphora geniculata*, Hymenoptera, Tenthredinidae, Nematinae, defense, exocrine secretion, ventral gland, *Sorbus*, benzaldehyde, borneol, bornyl acetate, mandelonitrile.

INTRODUCTION

There are 12 familes of sawflies with approximately 1000 described species in North America north of Mexico (Smith, 1979). Larvae of most of these primitive Hymenoptera feed on the young foliage of conifers, hardwoods, and other plants. Some are leafminers, gall-formers or wood- or stem-borers. If their populations reach outbreak levels, some species may become serious pests of forest and shade trees and cause significant economic damage (Coulson and Witter, 1984).

Species belonging to the Tenthredinidae are diverse in both habit and larval appearance. Some species are economically important, including the mountain

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ash sawfly, *Pristiphora geniculata*. Although not considered a forest pest, this sawfly damages ornamental plantings throughout Canada and the northeastern United States, reducing the esthetic value of the trees. The larvae are gregarious and feed from the edge of the leaf inward until all the leaf except the midrib has been consumed (Benson, 1950).

While feeding on their host plants, immature sawflies appear to be easy prey for potential predators. Benson (1950) clearly indicated that sawflies have evolved a variety of defensive strategies to protect their larval stages, including chemical mechanisms. These chemical defenses can be of two subcategories. When some species are disturbed, they regurgitate stored host plant oils. These include *Neodiprion sertifer* (Diprionidae) (Eisner et al., 1974) and the Australian species *Perga affinis* (Pergidae) (Morrow et al., 1976). Others, which belong to the Tenthredinidae (Nematinae), release exocrine secretions from medioventral abdominal glands (Benson, 1950).

Glandular openings in the ventral midline of abdominal segments 1–7, one behind each pair of prolegs on segments 2–7, are characteristic of one subfamily of sawflies, the Nematinae (Yuasa, 1922, Benson, 1950, Maxwell, 1955). When a larva is disturbed, it bends its abdomen over its head to expose the glandular openings. The defensive secretions are delivered from eversible glands.

In species with well-developed glands, the first and last glands are smaller than the others (Boevé and Pasteels, 1985, p. 1024). In describing the morphology of the abdomen of *Nematus spiraeae* (Nematinae: Tenthredinidae), Barlet (1982) reported that the abdomen has eversible glands on segments 1–7. The gland on the first segment is smaller and has a reduced musculature compared to the others. It may be that these morphological differences correlate with chemical differences as well (Jonsson et al., 1988).

Before the first reports on the chemistry of these secretions, Benson indicated that the secretions of some species smelled like benzaldehyde. Later analyses of these secretions have shown that they contain a variety of terpenoid alcohols and aldehydes, aliphatic acetates, benzaldehyde, and unsaturated aliphatic alcohols and aldehydes (Boevé et al., 1984; Jonsson et al., 1988). The relative concentration of components appears to be species specific.

In this paper we report the composition of the secretion of *Pristiphora geniculata*, commonly called the mountain ash sawfly. When they are disturbed, the larvae of this North American species discharge a secretion that has a menthol-like odor.

METHODS AND MATERIALS

Collection Data. Larvae were easily collected from the underside of foliage of mountain ash (*Sorbus* sp.) in Rochester, New York (Monroe County) during the third week of June 1988. Small branches containing leaves with

feeding groups of larvae were clipped from the limbs and placed in a container for transport to the laboratory.

The larvae were maintained in the laboratory for several days at room temperature. Fresh mountain ash leaves were added daily. The secretions were collected by gently placing each larva on a large sheet of filter paper. The head was gently probed with a filter paper square (1–2 mm^2) held with forceps, and the secretion was collected on a second filter paper square that was held with forceps in the other hand. The behavior of *P. geniculata* facilitated this method of collection because when disturbed, the larvae raised their abdomens over their thoraxes, thus exposing the ventral side where the glands open. After completing this procedure once or twice, the filter papers were placed in a glass vial containing methylene chloride. This procedure was repeated for each specimen. Samples were pooled such that each extract represented multiple collections from approximately 100 larvae.

As larvae were being collected, exuviae were frequently seen attached to defoliated branches. These cast cuticles were also extracted with methylene chloride. Fresh leaves of mountain ash and some of the frass that accumulated in the containers in the laboratory were also extracted with methylene chloride.

Chemical Analyses. The solvent was drawn off the filter paper squares after 24 hr and concentrated by air evaporation. Extracts were analyzed on a Finnigan MAT 4500B gas chromatograph–mass spectrometer utilizing a 30-m × 0.25-mm capillary fused silica column containing SPB-1 or SPB-5 (0.25 μm) temperature programmed from 60 to 300°C at 10°C/min. Retention times and mass spectra were compared with authentic compounds obtained commercially or synthesized by standard methods.

RESULTS

The compound eluting first exhibited a molecular ion at *m/z* 106 with additional ions at 105, 77, and 51. It was identified as benzaldehyde (32%). The second compound (8%) exhibited a weak molecular ion at 154 with additional ions at 139(10), 136(15), 121(15), 96(10), 95(100), 93(20), 84(5), 83(7), 82(10), 71(7), 69(10), 67(15), 55(15), 43(20), and 41(25) and was identified as borneol. The third component (28%) exhibited a molecular ion at 196(0.5) with additional ions at 154(8), 136(28), 112(30), 108(20), 95(85), 80(20), 43(100) and 41(43). It was identified as bornyl acetate by comparison of its retention time and spectrum with those of synthetic bornyl and isobornyl acetates. A small peak (2%) eluting after the bornyl acetate had an odd molecular ion at *m/z* 133 with additional ions at 132, 106, 105, 77, and 51. An authentic sample of mandelonitrile exhibited the same spectrum and retention time as that of the natural product. Three additional components were identified as tetradecyl (2%), hexadecyl (9%), and octadecyl acetate (19%) by comparison with authentic samples.

Extracts of shed exuviae contained benzaldehyde, borneol, bornyl acetate, tetradecyl acetate, hexadecyl acetate, and octadecyl acetate in almost the same proportions as the secretion. Extracts of frass and mountain ash leaves showed none of the volatiles listed above.

DISCUSSION

The chemical composition of the larval exocrine secretions of species belonging to the Nematinae (Tenthredinidae) appear to be chemically diverse. Eighteen compounds have been isolated from the exocrine exudates of 11 species. Each secretion contains from one to eight volatile compounds. Four species release secretions that show only one compound. For example, exudates of *Croesus septentrionalis* and *C. varus* (Boevé et al., 1984) contain *cis,trans*-dolichodial. With the exception of these, all the other species exhibit species-specific secretions.

Benzaldehyde is found in the secretion of seven of the species investigated and appears to be the most common compound found in sawflies. It is also frequently observed in other arthropod exocrine secretions (Blum, 1981). For example, benzaldehyde is found in the defensive secretion of a variety of species of polydesmid millipedes (for a review see Blum, 1981) as well as in centipedes (Jones et al., 1976). It occurs in the mandibular glands of bees (for review see Wheeler and Duffield, 1988), in the mandibular glands of the ant, *Veromessor* (Blum et al., 1969), and in the anal gland secretion of the tropical ant, *Azteca* (Blum, 1981). Benzaldehyde also occurs in the defensive secretions of species in several families of Coleoptera (Blum, 1981).

The release of benzaldehyde in millipedes is accompanied by the liberation of hydrogen cyanide (HCN). It has been shown that HCN in millipedes can be produced from several different precursors. For example, HCN is produced from *p*-isopropylmandelonitrile glycoside in *Polydesmus vicinus* (Pallares, 1946); from mandelonitrile benzoate in *Polydesmus collaris collaris* (Casnati et al., 1963); from mandelonitrile glycoside in *Pachydesmus crassicutis* (Blum and Woodring, 1962); and from mandelonitrile in *Apheloria corrugata* (Eisner et al., 1963) and *Oxidus gracilis* (Towers et al., 1972).

In insects the biosynthetic pathways for benzaldehyde are unknown, although phenylalanine is presumed to be the precursor. The intermediates have not been demonstrated. It is interesting to find both mandelonitrile and benzaldehyde in the exocrine secretion of *P. genticulata*. Although benzaldehyde is found in the larval secretions of six other species of sawflies (Boevé et al., 1984; Jonsson et al., 1988), mandelonitrile has not been reported. This is the first direct identification of mandelonitrile in sawfly larval exocrine secretions by gas chromatographic–mass spectroscopic analysis. Traces of mandelonitrile

have been detected in millipedes by employing reagents that are selective for specific functional groups, (i.e., cyanide or aldehyde). In arthropods, Eisner et al. (1963) showed that millipedes have a two-chambered gland in which, they hypothesized, the mandelonitrile mixes with an enzyme in the second chamber, causing the liberation of HCN and benzaldehyde as well as other products. Benzaldehyde has been identified in 18 species of millipedes (Duffey et al., 1977); no mandelonitrile was detected, although mandelonitrile benzoate and benzoyl cyanide were found in some of the species. Experiments by Duffey (1981) demonstrated the presence of an enzyme, α-hydroxynitrile lyase, in the second chamber of the gland, with sufficient activity to account for the coproduction of benzaldehyde and hydrogen cyanide from mandelonitrile in millipedes. The form of mandelonitrile stored in the gland, whether as the free compound or as a glycoside, appears to vary among millipedes. Because the mandelonitrile is converted immediately to benzaldehyde and hydrogen cyanide, mandelonitrile would not be expected to be a dominant component of polydesmoid millipede defensive secretions. However, it does not rule out that it could be a trace component. Mandelonitrile has been previously reported by Blum et al. (1981) from the pygidial gland secretion of a species of tiger beetle.

Although mandelonitrile is a minor component in the *P. geniculata* secretion, it was reproducibly seen. It did not disappear upon storage. We did experience the problem of instability during gas chromatographic (GC) analysis cited by other authors (Eisner et al., 1963; Jones et al., 1976; Conner et al., 1977; Blum et al., 1981) but only on certain columns. Over 50% of authentic mandelonitrile, in fact, survived upon analysis by capillary GC-MS using a SPB-1 column. It did not survive at all on a SPB-5 column.

The presence of mandelonitrile in the defensive secretion of *P. geniculata* and possibly of other species of sawflies that produce benzaldehyde may reflect a fundamental difference between polydesmoid millipedes and these nematine sawflies. The sawflies may lack the hydronitrile lyase in their defensive glands that the polydesmoids employ to convert mandelonitrile to benzaldehyde and hydrogen cyanide when disturbed.

Borneol has been found previously in two species of *Formica* ants along with isoborneol (Bühring et al., 1976). It was isolated from extracts of combined heads and thoraces. Both borneol and bornyl acetate have been identified in the ventral gland extracts of a European species of sawfly, *Pristiphora erichsonii* (Jonsson et al., 1988). Bornyl acetate has also been previously isolated from an opilionid (Epka et al., 1984).

The C_{14} and C_{16} acetates have been identified from the pygidial gland secretion of the tiger beetle *Cicindela flexuosa* (Carabidae) (Hefetz et al., 1984). Tetradecyl, hexadecyl, and octadecyl acetates have been previously identified in the Dufour's gland secretion of several genera of formicine ants including *Formica* (Regnier and Wilson, 1971; Bergström and Löfqvist, 1973), *Lasius*

(Bergström and Löfqvist, 1970), and *Camponotus* (Bergström and Löfqvist, 1971, 1972). In the ants, these compounds serve behavioral roles in releasing alarm-defensive behaviors.

There has been some discussion of whether the chemistry of all abdominal gland secretions of sawflies is the same since the morphology may vary. Jonsson et al. (1988), in studying larvae of *Pristiphora erichsonii* and *P. wesmaeli*, reported collecting secretions only from the noneversible gland on the first abdominal segment. They used 3.0 × 12-mm pieces of filter paper held with forceps to collect the larval secretion. Since we have found by experience that many of the larvae twist and move during the collection process, it would seem very difficult not to contaminate the large filter paper pieces they used with secretion from the eversible glands on segments 2–7. In fact, they do suggest that the benzaldehyde that they found may have been contamination from glands 2–7. Although Jonsson et al. state that Boevé et al. (1984) and Boevé and Pasteels (1985) collected secretion only from glands 2–6, we cannot find such a statement in either paper. Boevé and Pasteels (1985) do state that glands 2–6 were used for their morphological measurements since glands on abdominal segments 1 and 7 are smaller than the others. Furthermore, Barlet (1982), in an investigation of the glandular morphology of *Nematus spiraeae*, found that the only differences in the gland on segment 1 is that it is smaller and exhibits a reduced musculature. This is also one of the species whose chemistry was reported by Boevé et al. (1984). A resolution of the question of functional differentiation between the gland from the first abdominal segment and the others awaits comparison of the chemistries from both types from the same laboratory.

The material analyzed by us appears to come from all seven abdominal glands. As the secretion was being collected, four to six "darkened spots" frequently appeared on the filter paper, suggesting that the larvae everted several or all the glands and may actually have ejected or squirted small droplets of exocrine secretion. By twisting and "snap bending," the larvae may hurl droplets in the direction of the stimuli. There is no question that they direct the openings of the gland toward the direction of a stimulus.

Jonsson et al. suggest that the benzaldehyde in their secretion may come from contamination from glands 2–7. We apparently collected material from all seven glands, and the composition of our secretion is essentially the same as found for *P. erichsonii* (with the absence of myrtenol and *trans*-pinocarveol but the presence of mandelonitrile). We assume that the secretion of *P. geniculata* is from all seven abdominal glands since no attempt was made to differentiate between glands. With our species it would be a formidable task to collect only the exocrine products from the first gland without contamination from the others. Totally different collecting procedures would have to be employed.

The fact that the exuviae contain the same defensive compounds as the larval secretions may account for the fact that the larvae eat the exuviae only

of the first instar, leaving the subsequent ones intact (Forbes and Daviault, 1964).

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SEASONAL PATTERNS OF JUGLONE IN SOIL BENEATH *Juglans nigra* (BLACK WALNUT) AND INFLUENCE OF *J. nigra* ON UNDERSTORY VEGETATION

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Abstract—The allelopathic nature of *J. nigra* L. was investigated in several planted mixed hardwood stands located near Syracuse, New York. Concentrations of chloroform-extracted juglone from soil collected beneath *J. nigra* was determined by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). Soil juglone concentrations were corrected based on recovery of synthetic juglone added to soil. Soil juglone levels were high in the spring, decreased during the summer, and were high again in the fall. The quantification of juglone from soil by HPLC was found to be more accurate than by TLC. Regression analysis indicated that individual tree variation in soil juglone levels could not be explained by differences in soil moisture, pH, organic matter content, and texture. The results of juglone recovery experiments suggest that chloroform-extractable juglone does not persist in soil. Juglone degradation by microorganisms could only explain a portion of the juglone decline. Ordinations revealed that the herbaceous and woody vegetation beneath *J. nigra*, in comparison to vegetation beneath *Acer saccharum* and *Quercus rubra*, is distinct in only one of the four stands studied. This vegetational difference did not appear to be a consequence of any strong allelopathic influences of *J. nigra* (Scheffé's method of contrast, chi-square analysis). The allelopathic nature of juglone under these field conditions is questionable.

Key Words—Allelopathy, black walnut, *Juglans nigra* L., juglone, 5-hydroxy-1,4-naphthoquinone.

INTRODUCTION

Juglans nigra L. (black walnut) is a putatively allelopathic species (e.g., see Rice, 1984) despite the paucity of quantitative proof. The earliest account of the injurious effects of *Juglans* spp. on other plant species dates to 23–79 AD by the Roman natural historian Pliny the Elder (Gries, 1943; Willis, 1985). It was not until 1928 that Davis (1928) attributed the toxicity of *J. nigra* to the napthoquinone juglone (5-hydroxy-1,4-naphthoquinone) as suggested by Massey (1925). The toxic nature of *Juglans* spp. was first doubted during the 17th century by Evelyn (Gries, 1943). More recent studies have both supported and rejected the toxic effects of *Juglans* spp. (see reviews by MacDaniels and Muenscher, 1941; MacDaniels and Pinnow, 1976).

Most studies have investigated the influence of *J. nigra* on various vegetables, field crops, fruit trees and ornamental species (Cook, 1921; Massey, 1925; Schneiderhan, 1927; Pirone, 1938; Reinking, 1943; Strong, 1944; Brooks, 1951; Sherman, 1971; MacDaniels and Pinnow, 1976). Since *J. nigra* is a very valuable timber species, cocrop and nurse-crop species that will increase overall yield have been sought. Allelopathic influences of *J. nigra* have been implicated in failures to establish mixed *J. nigra* plantations (Schreiner, 1949; von Althen, 1968; Wiant and Ramirez, 1974; Gabriel, 1975) and in old-field succession (Bratton, 1974). Several studies have tested the toxic effects of juglone on species considered for coplanting with *J. nigra* (Rietveld, 1981, 1983; Rietveld *et al.*, 1983; Ponder and Tadros, 1985).

Numerous species (e.g., tomato, alfalfa, and pine) have been reported to be negatively influenced by *J. nigra* (Brooks, 1951; Sherman, 1971; MacDaniels and Pinnow, 1976; Rietveld, 1981; Boes, 1986). However, the growth and survival of many species are unaffected or even promoted by *J. nigra* (Brooks, 1951; Boes, 1986). Black raspberry (*Rubus occidentalis*) and Kentucky bluegrass (*Poa pratensis*) were found consistently more often within than beyond the root spread of *J. nigra* (Brooks, 1951). *J. nigra* has even been found to improve forage cover in southeastern Ohio pastures (Smith, 1942). Also, low concentrations of juglone prove to be stimulatory in some bioassays (Funk *et al.*, 1979; Rietveld, 1981, 1983; Rietveld *et al.*, 1983; Dawson and Seymour, 1983). The allelopathic nature of *J. nigra* has been questioned because of the contradictory claims of species affected (Greene, 1930; Davidson, 1939; MacDaniels and Muenscher, 1941; Mattoon, 1944). For example, studies have found *J. nigra* to be detrimental to pines (Schreiner, 1949; von Althen, 1968; Wiant and Ramirez, 1974), a claim that is inconsistent with the findings of Camp (1986). Rietveld (1981) and Fisher (1978) report pines growing, both well and poorly, in mixed plantings with *J. nigra*.

Although there have been many reports of juglone's effects on various plant species, only two studies have investigated the relationship between jug-

lone concentration in the soil and its effect on plant growth in the field (Ponder and Tadros, 1985; Ponder, 1987). Yet these field studies on vegetation response and patterns in relation to soil juglone concentrations, and the edaphic factors affecting juglone's persistence, are necessary in order to achieve a better understanding of *J. nigra* allelopathy.

The purpose of this research was to evaluate the allelopathic nature of *J. nigra* in several planted mixed hardwood stands located near Syracuse, New York. The seasonal pattern of juglone in soil beneath *J. nigra* and the factors affecting the persistence of juglone in soil were investigated. The influence of *J. nigra* on the understory vegetation was also examined.

METHODS AND MATERIALS

Study Site. The mixed *J. nigra* plantations used in this study are located at the State University of New York College of Environmental Science and Forestry (SUNY-CESF), Lafayette Road Experiment Station near Syracuse, New York. Plantations were established in 1914. Early stand history is discussed elsewhere (Prichard, 1941). Four areas were chosen for study as follows (letters refer to blocks shown in Prichard, 1941): Sites L and G consist primarily of *J. nigra* and *Quercus rubra* L. Site E1 consists primarily of *J. nigra, Acer saccharum* Marsh. and *Q. rubra*. Site E1 was divided into two sites designated as A (west side of trail, absence of *Q. rubra*), and B (east side of trail). Although the principal soil types are Wassaic silt loam and Benson-Wassaic Rock outcrops (U.S. Department of Agriculture, 1972), the soil is very sandy in some locations that were previously occupied by a postglacial lakeshore (Prichard, 1941).

Soil Juglone Quantification. Soil samples were collected on April 21, 1987, July 16, 1987, and November 15, 1987, from around six *J. nigra* in site B and throughout a *Q. rubra* and *A. saccharum* stand located adjacent to site B (referred to as control stand). Soil samples collected within the control stand were at least 15 m from the nearest *J. nigra* in site B. A soil sampling tube was used to collect soil in an area between 0.5 and 1.0 m from the outside edge of the tree base, at a depth of 10 cm. A composite sample of 12 soil cores was collected around each tree. A composite sample of approximately 40 soil cores was collected from the control stand. Soil samples were placed in plastic bags and immediately transported to the laboratory where they were stored at 4°C for no longer than 24 hr until extraction.

In the laboratory, the soil was sifted through a 10-mesh (2-mm) sieve to remove pebbles and organic debris. The high moisture content of some samples prevented sieving, leading to the manual removal of the large pebbles and organic debris. The juglone extraction procedure was modified from Ponder and

Tadros (1985). One hundred grams of soil was extracted with 100 ml chloroform by shaking for 1 hr. The mixture was vacuum-filtered through Whatman qualitative No. 1 filter paper. The soil was reextracted with 50 ml chloroform for 30 min. The filtrates were combined and reduced *in vacuo* at 30°C to approximately 3 ml. The concentrated extract was dried under N_2.

The dried extract was resuspended in 0.5 ml of chloroform. Aliquots were streaked onto a silica gel G TLC plate (Fisher Scientific, Pittsburgh, Pennsylvania) and developed with hexane–chloroform–glacial acetic acid (140:40:20). A juglone standard (Aldrich Chem. Co., Milwaukee, Wisconsin) was run on each plate. The putative juglone band was eluted with chloroform. Each sample was scanned on a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. Juglone was quantified using the absorbance at 427 mm based on a standard curve of juglone in chloroform.

Juglone was also quantified by HPLC after TLC purification. The dried sample was resuspended in 0.7 ml MeOH to which 0.3 ml of 0.1 N acetic acid was added after approximately 1 min. The solution was centrifuged through 0.45-μm nylon filters (Rainin Instrument Co. Inc., Woburn, Massachusetts). The juglone samples were further purified and quantified by HPLC (model 2350 Isco, Inc., Lincoln, Nebraska) on a 5-μm ODS column (4.6 × 150 mm). Elution was isocratic with methanol containing 30% 0.1 N acetic acid at 0.8 ml/min. The juglone peak was collected and partitioned into chloroform. The identity of the juglone peak was confirmed by GC-MS on a Finnigan 4000 GC-MS-DS system. The GC was performed on a SPB-1 fused silica capillary column (8 m × 0.25 mm ID) with a film thickness of 0.25 mm (Supelco, Bellefonte, Pennsylvania). The carrier gas was helium at 1 ml/min. The oven temperature was raised 10°C/min from 50°C to 280°C and held for 10 min. Mass spectrometry was by electron impact at 70 eV.

Juglone quantification by HPLC was based on a juglone standard curve of peak areas monitored spectrophotometrically at 427 nm (De Scisciolo, 1988). The soil juglone concentrations were corrected according to a standard curve of juglone recoveries from soil (see Recovery Experiments section) (De Scisciolo, 1988).

The 95% confidence intervals for paired samples (paired *t* tests) were used to test the signficance of the temporal changes in soil juglone levels, the differences between the TLC and HPLC methods of juglone analysis, and the effect of long-term soil storage on chloroform extractable juglone levels (Wonnacott and Wonnacott, 1985).

Recovery Experiments. For the juglone recovery experiments, the method of juglone analysis was as previously described except that a solution of commercial juglone in chloroform was added to 100 g of sieved soil collected from the control stand. After 10 min, the soil was extracted with chloroform. Soil differing in physical and chemical properties from that of the SUNY Experiment

Station was collected from the Albany Pine Bush for comparison of juglone recovery. For the sterilization experiments, 100 g of control soil was autoclaved for 30 min at 121°C. Commercial juglone in chloroform was added to the cooled soil and extracted after the specified incubation periods.

For juglone recovery using water as the extracting solvent, commercial juglone in chloroform was added to 100 g of control soil in a 500-ml Erlenmeyer flask. After 10 min, 150 ml distilled water was added and the mixture was shaken for 1.5 hr. The slurry was centrifuged (Sorvall RC-5B Refrigerated Superspeed Centrifuge), the water decanted, and the water vacuum-filtered through a Buchner funnel with Whatman qualitative No. 1 filter papers. The water extract was partitioned against chloroform, the chloroform fraction was concentrated 3 ml *in vacuo*, and then dried under N_2. The subsequent steps for juglone quantification were as described.

Analysis of Soil Properties. For gravimetric soil moisture analysis, 15 g of sifted, field-collected soil were dried at 105°C for 48 hr and then reweighed. Percent moisture content was expressed as a percentage of the oven-dry weight (Wilde *et al.*, 1979). For all subsequent soil analyses, air-dried soil was used. pH was determined using a Fisher Accumet pH-meter model 805 MP with a gel filled electrode, according to the method of Wilde *et al.* (1972). Percent organic matter was determined by the method of wet decomposition oxidation as described in Richards (1969). Soil texture was determined by the hydrometer method described in Wilde *et al.* (1979).

Scheffé's method of contrast was used to compare differences in soil properties between samples (Wonnacott and Wonnacott 1985). Single and multiple regression analyses were used to determine any relation between juglone levels and each of the measured soil properties. Paired *t* tests were used to test the significance of the temporal changes in percentage of soil moisture (Wonnacott and Wonnacott, 1985).

Vegetation Study. The understory vegetation (i.e., those woody and herb species that occur below the main tree canopy) was chosen as a field bioassay to assess the allelopathic potential of *J. nigra*. Four 0.25-m^2 square plots were positioned to the north, south, east, and west around each selected *J. nigra*, *Q. rubra*, and *A. saccharum*, 0.5 m from the base. The vegetation sampled was thus in the area between 0.5 and 1.0 m from the tree base. Control plots (0.25 m^2) were systematically located in areas near the sample tree but at least 3 m away from any tree base. The numbers of sample trees and control plots at each site were as follows: A—5 *J. nigra*, *A. saccharum*, and control plots; B—12 *J. nigra*, 15 *A. saccharum*, 8 *Q. rubra*, 13 control plots; G—4 *J. nigra*, *Q. rubra*, 6 control plots; L—3 *J. nigra*, *Q. rubra*, and control plots.

Understory vegetation was sampled both in the spring (May 10, 1986) and in the summer (July 17, 1986). Voucher specimens have been deposited in the SUNY-CESF herbarium. Nomenclature for vascular species follows Gleason

and Cronquist (1963). The number of individuals of each species (i.e., density) was recorded in each plot. Percent cover for each species was estimated for those individuals ≤ 0.6 m in height. Density and percent cover for each species were figured per square meter for each sample tree and control plot.

Scheffés method of contrast was used to compare mean density and percent cover for each plot type (Wonnacott and Wonnacott, 1985). For subsequent vegetation analyses, only understory species with a frequency ≥ 2 were considered. Contingency tables (2×2) were used to reveal associations between understory species and plot type (Sokal and Rohlf, 1981). Multivariate analyses of density and percent cover data were performed using PC-ORD software (McCune, 1987). For Bray-Curtis ordination (BC-ORD), the distance measure, endpoint selection, and projection geometry options chosen were Sorensen coefficient, variance-regression, and Euclidean, respectively, as recommended by Beals (1984). The data matrices were relativized within samples using general relativization (McCune, 1987) as recommended by Beals (1984). Detrended Correspondence Analysis (DECORANA) ordinations were also run for comparison with the Bray-Curtis ordinations. Two-way Indicator Species Analysis (TWINSPAN) was used to complement the Detrended Correspondence Analysis ordination as suggested by Kershaw and Looney (1985).

RESULTS

Soil Juglone Analysis. Juglone levels in soil beneath *J. nigra* varied throughout the season at the SUNY Experiment Station study site (Figure 1). Mean juglone levels in the summer samples were significantly lower than in both the spring and fall samples (according to the 95% confidence intervals using paired *t* tests, data not shown). The slightly lower juglone levels in the fall compared to the spring were not significant (paired *t* test). The temporal pattern of soil moisture beneath *J. nigra* was similar (Figure 2), although the differences between sampling periods were not significant (using paired *t* tests).

The physical and chemical properties of soil beneath *J. nigra* in site B differed among the six samples (Table 1). There was little correlation of soil juglone levels with any of the measured soil variables. Multiple regression analysis was performed to investigate the relationship between soil juglone levels with soil variables in combination, but the results were inconclusive because of an inadequate sample size.

Comparison of Juglone Quantification Methods. After the TLC step in the purification of soil-extracted juglone, a yellow precipitate collected on the nylon filters. The yellow precipitate was not observed when filtering juglone standards but was seen in field soil samples even when juglone was not present. The spectrum of this precipitate dissolved in chloroform was not indicative of jug-

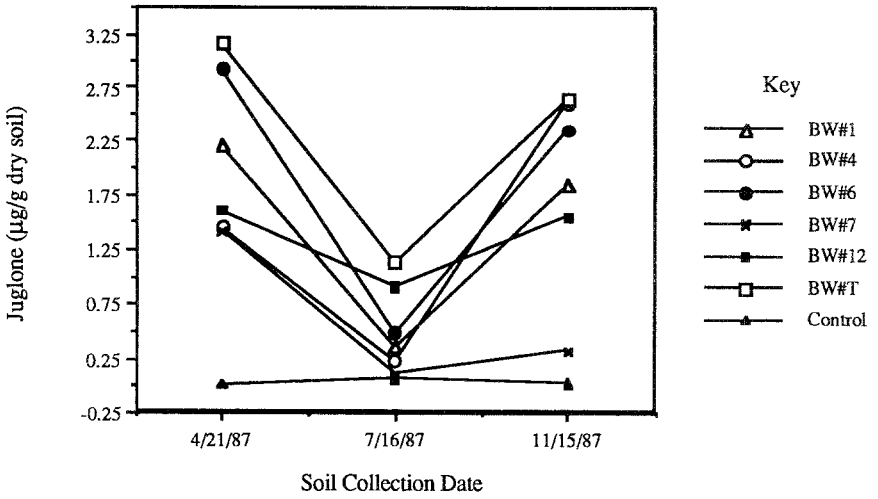

FIG. 1. Temporal pattern of juglone in soil (quantified using HPLC), beneath *Juglans nigra* and in control area from site E1B. The juglone concentrations are corrected based on standard curves of the recovery experiments. Values for 4/21/87 sample are based on one determination with a slightly modified procedure from the 7/16/87 and 11/15/87 data (see Materials and Methods section for details). Values for 7/16/87 and 11/15/87 are means based on two to five determinations with the exception of BW#1, 7/16/87, which is based on only one determination.

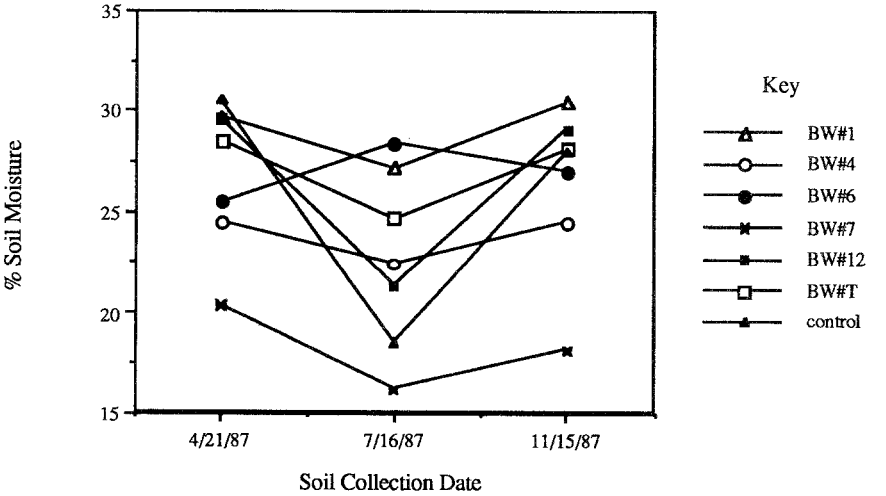

FIG. 2. Temporal pattern of soil moisture beneath *Juglans nigra* and in control area from site E1B. Values are based on one determination with the exception of BW#4, 7/16/87 which represents the mean of three determinations.

TABLE 1. PHYSICAL AND CHEMICAL PROPERTIES OF SOIL SAMPLES[a]

Soil sample	pH	Organic matter (%)	Clay (%)	Silt (%)	Sand (%)
E1B BW#1	6.7 ab	3.89 c	19 b	31 bc	50 b
E1B BW#4	6.7 ab	2.89 b	14 a	22 b	65 d
E1B BW#6	6.8 b	3.18 b	19 b	27 b	54 c
E1B BW#7	7.4 c	2.28 a	12 a	14 a	73 e
E1B BW#12	6.6 a	4.27 c	18 b	34 c	48 ab
E1B BWT	6.6 a	3.89 c	20 b	35 c	46 a
E1B Control	6.5	4.24	26	40	34

[a] Determinations represent averages of six replicates for pH and percent organic matter, three replicates for soil texture. Means within a column followed by the same letter are not significantly different at the 0.05 level according to Scheffé's method of contrast.

lone, but the precipitate did quantify as juglone in the spectrophotometric determination reported by Ponder and Tadros (1985). The quantification of this yellow impurity as juglone at the TLC step of juglone purification probably explains the significant difference in juglone levels between the two methods (Table 2). The amount of this impurity in soil extracts was relatively constant as judged by the similar differences of the means, with the exception of 4/21/87 r1, which shows a greater loss in juglone, probably because of streaking a smaller amount of the total soil extract onto the TLC plate.

Recovery Experiments. The recovery of juglone standards added to soil and extracted within 10 min with chloroform varied greatly between the soil collection periods (Table 3). Juglone recoveries increased with large juglone

TABLE 2. COMPARISON OF SOIL JUGLONE QUANTIFICATION AFTER TLC AND HPLC

		Mean juglone concentration (μg/g dry soil)		
Soil collection date	Sample size	TLC[a]	HPLC	Difference of the means
4/21/87 r1[b]	7	1.41	0.98	0.42 *[d]
4/21/87 r2[c]	7	0.69	0.48	0.21 *
7/16/87	19	0.51	0.28	0.23 *
11/15/87	16	1.73	1.48	0.25 *

[a] Juglone quantified using a spectrophotometer, wavelength = 427 nm.
[b] r1 = modified procedure: 0.04-ml aliquots of soil extract streaked onto TLC plate (see Materials and Methods).
[c] r2 = soil samples stored in plastic bags at −18°C for seven months.
[d] Difference of the means are statistically significant according to the 95% confidence intervals for paired samples.

TABLE 3. JUGLONE RECOVERY FROM SOIL AFTER 10-MIN INCUBATION USING CHLOROFORM AS EXTRACTING SOLVENT[a]

Soil collection date	Sample size	Juglone (μg) added to 100 g control soil	Recovery (%)	
			TLC[b]	HPLC
4/21/87[c]	1	50	60	20
	1	100	70	29
	1	200	78	51
	1	100 NS[d]	77	58
7/16/87	3	50	99	49
	2	100	81	50
	2	200	82	64
	2	100 NS	86	81
11/15/87	2	50	82	49
	2	100	83	72
	3	200	83	82
	2	100 NS	83	89
	2	100 dry[e]	126	73

[a] For sample size >1, recoveries represent mean values.
[b] Juglone quantified using a spectrophotometer, wavelength = 427 nm.
[c] For 4/21/87 recoveries 40 μl of total 500 μl concentrated soil extract was spotted onto TLC plate; other recovery experiments used 200 μl of sample spotted onto TLC plate.
[d] NS = no soil, juglone added to empty flask and taken through procedure.
[e] dry = soil air dried, concentrated soil extract 1000 μl, 200 μl spotted onto TLC plate.

additions. Recovery of 200 μg of juglone added to soil and extracted with water was $<1\%$.

The recovery of juglone dropped substantially after 4 hr of incubation at room temperature in both sterile and nonsterile field soil (Figure 3). After 43 hr, less than 1% of the added juglone was recovered (data not shown). Juglone extracted from moist field soil collected July 16, 1987, from beneath *J. nigra* BW#6 contained 0.23 μg juglone/g dry soil, but when the soil was air-dried for two days, only 0.03 μg juglone/g dry soil could be extracted. Extractable soil juglone dropped significantly upon storage in plastic bags at $-18°C$ for seven months.

Juglone recovery was higher and had a slower rate of decline in sterile soil as compared to nonsterile soil (Figure 3). Soil collected from the Albany Pine Bush had the highest initial juglone recovery and slowest rate of decline.

Vegetation Study. Juglans nigra had no negative influence upon the mean density and percent cover of understory vegetation compared to *A. saccharum, Q. rubra,* and control plots (Table 4). In fact, the density of understory vegetation beneath *J. nigra* was significantly higher than beneath *Q. rubra* and con-

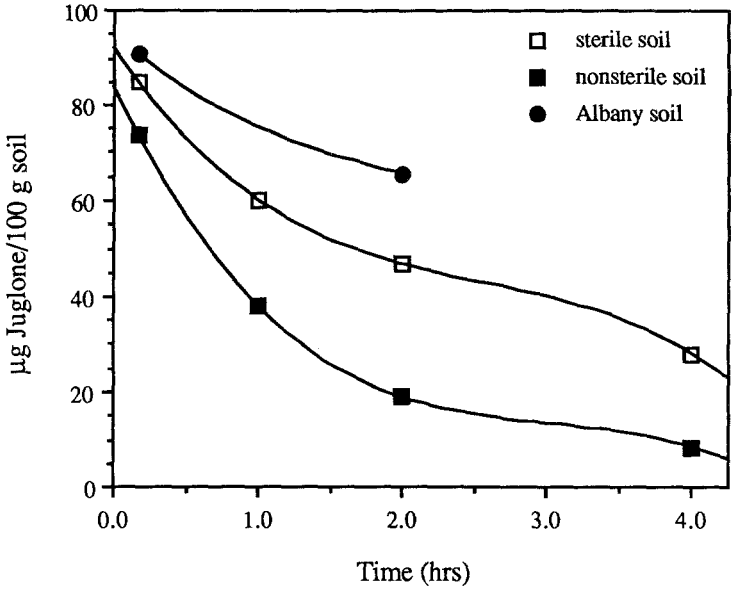

FIG. 3. Comparison of juglone recovery from sterile and nonsterile field soil collected 10/18/87 from control area in site E1B (soil moisture = 27%, pH = 6.61, percent organic matter = 4.65, texture = 28%, 39%, 33%, clay, silt, and sand, respectively), and nonsterile field soil collected 11/27/87 from the Albany Pine Bush, Albany, New York (soil moisture = 5.1%, pH = 5.60, percent organic matter = 0.41, texture = 3%, 2%, 95%, clay, silt, and sand, respectively). Juglone standards added to soil were extracted after the specified incubation periods. Juglone was quantified using HPLC.

TABLE 4. MEAN-DENSITY AND PERCENT COVER OF UNDERSTORY VEGETATION[a]

Plot type	May, site B (% cover/m^2)	July, site B (% cover/m^2)	July, site L (density, N/m^2)	July, sites A, B, G, L (% cover/m^2)
Acer saccharum	8.8 ab	11.8 ab		11.1 a
Juglans nigra	10.5 ab	19.7 ab	48.3 b	29.2 ab
Quercus rubra	2.7 a	5.0 a	14.2 a	21.8 ab
Control plots	17.7 b	25.2 b	13.3 a	36.5 b

[a]Only those sites and sampling dates which resulted in statistically significant relationships among overstory trees and control plots are presented. Means within a column followed by the same letter are not significantly different at the 0.05 level according to Scheffé's method of contrast.

trol plots at site L in July. The percent cover of vegetation beneath *Q. rubra* at site B and beneath *A. saccharum* for all combined sites in July is significantly less than that of control plots.

Specific association patterns of overstory *J. nigra* trees with the understory vegetation showed a negative relationship only with *A. saccharum* seedlings and saplings (Table 5). *Rubus occidentalis, Circaea quadrisulcata,* and *Dryopteris spinulosa* are positively associated with *J. nigra.*

Since Two-way Indicator Species Analysis results were not as useful for revealing pattern in the understory vegetation, only results from the Bray-Curtis (BC) and Detrended Correspondence Analysis (DCA) ordinations (and only May and July data) are presented (Figures 4, and 5). For the May sample (Figure 4), density values were ordinated instead of percent cover because of the low cover for all species at this early time. There is a strong separation of overstory trees and control plots according to site. Plots in sites G and L are similar to each other and most dissimilar to plots in sites A and B. Site B *J. nigra* plots are distinct from all others.

For the July sample (Figure 5), percent cover values were ordinated instead of density because of the inability to obtain density values for *Parthenocissus quinquefolia.* Again, there is a distinct separation of plots in sites L and G from

TABLE 5. CHI-SQUARE ANALYSIS OF UNDERSTORY VEGETATION DATA FROM SITES A, B, G, AND L COMBINED[a]

Understory species	Plot type			
	A. saccharum	*J. nigra*	*Q. rubra*	Control
May data				
Acer saccharum	+	−	0	0
Fraxinus spp.	+	0	0	0
Geum canadense	0	0	0	+
Parthenocissus quinquefolia	−	0	0	0
Rhamnus cathartica	0	0	+	0
Rubus occidentalis	0	+	−	0
July data—similar to May data set except for the following additions:				
Circaea quadrisulcata	−	+	0	0
Dryopteris spinulosa	0	+	0	0
Prunus virginiana	+	0	0	0

[a] Additional species present in the understory (≥2 occurrences) which showed no association patterns: *Agrimonia gryposepala, Cornus alternifolia, C. florida, C. racemosa, Eupatorium rugosum,* grass spp., *Lonicera tatarica, Picea abies, Podophyllum peltatum, Polygonum virginianum, Prunus serotina, Toxicodendron radicans,* and *Veronica serpyllifolia.*

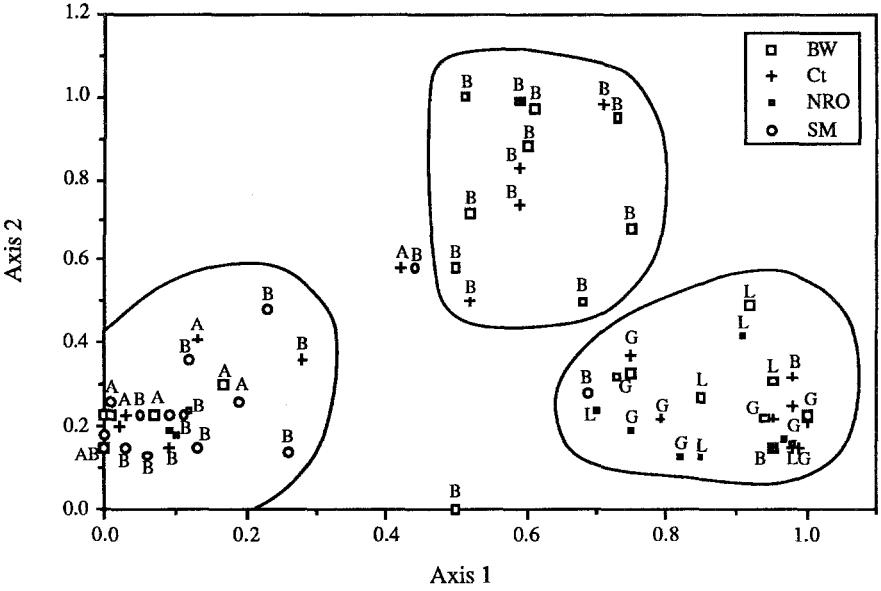

FIG. 4. Bray-Curtis ordination of the understory vegetation (density values, May sampling) beneath *A. saccharum* (SM), *J. nigra* (BW), and *Q. rubra* (NRO) trees and control plots (Ct). The overlaid letters A, B, G, and L refer to the respective sites. The encircled areas indicate related groups of plots.

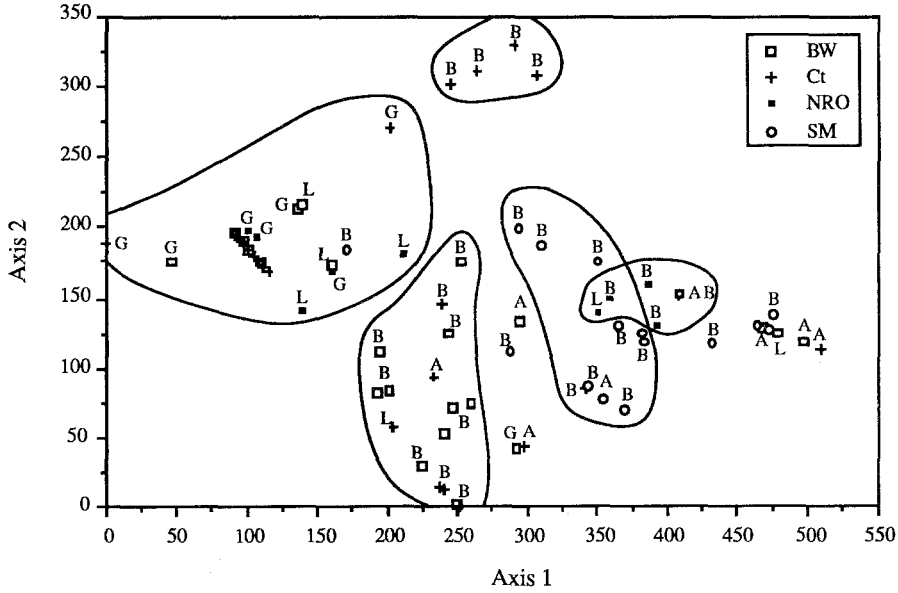

FIG. 5. Detrended correspondence analysis ordination of the understory vegetation (percent cover, July sampling) beneath *A. saccharum* (SM), *J. nigra* (BW), and *Q. rubra* (NRO) trees and control plots (Ct). The overlaid letters A, B, G, and L refer to the respective sites. The encircled areas indicate related groups of plots.

those in sites A and B. Within site B, four separate groupings of plots emerge according to overstory species, including a distinct group of *J. nigra* plots.

DISCUSSION

Soil Juglone Analysis. It is unclear whether the seasonal pattern of juglone present in soil beneath *J. nigra* at the SUNY Experiment Station (Figure 1) is caused by a seasonal pattern of juglone input or a seasonal pattern of juglone degradation or a combination of both. Temporal patterns of juglone in various *J. nigra* tissues is well documented (Lee and Campbell, 1969; Graves *et al.*, 1979; Hedin *et al.*, 1979; Coder, 1983; Cline and Neely, 1984), but the temporal contribution of juglone to soil from each plant part is unknown. If leaching of aboveground *J. nigra* tissues is responsible for substantial juglone input into the soil, then temporal soil juglone patterns should reflect precipitation patterns. Soil juglone and moisture seem to follow a similar temporal pattern (Figures 1 and 2). Alternatively, the observed temporal pattern of juglone in soil could be a result of temporal differences in juglone degradation. The low levels of soil juglone found during the summer (Figure 1), when soil moisture was lowest (Figure 2), are consistent with the conclusions of MacDaniels and Muenscher (1941) and Rietveld (1981), who suggest that the chemical breakdown of juglone is increased in well-aerated soil.

The endproducts of juglone degradation have been largely unexplored. Juglone is known to be a strong oxidizing agent (Gries, 1943; MacDaniels and Pinnow, 1976; Rietveld, 1981), suggesting that juglone becomes either reduced to a-hydrojuglone or broken down into other nontoxic substances (Gries, 1943). Juglone added to aqueous extracts of *C. illinoensis* leaves was found to decrease substantially within 2 hr with only a small fraction of the juglone being converted first to a-hydrojuglone and then to a compound thought to be a tetrahydrojuglone (Hedin *et al.*, 1980). Recently, a bacterium (*Pseudomonas putida* J1), isolated from soil beneath *J. nigra*, was found to metabolize juglone when grown with glucose (Rettenmaier *et al.*, 1983). Juglone was rapidly converted to 3-hydroxyjuglone and then slowly to 2,3-dihydroxybenzoate using resting cell experiments and further converted to 2-hydroxymuconic acid semialdehyde using crude extracts of cells (Rettenmaier *et al.*, 1983).

Many edaphic factors affecting allelochemical accumulation in soil such as pH, texture, and organic matter (del Moral and Muller, 1970; Dalton *et al.*, 1983; Oleszek and Jurzysta, 1987) have not been investigated for juglone. The differences in soil juglone levels beneath the six *J. nigra* trees in this study could not be explained by the variation in the measured edaphic factors. This failure to correlate soil properties with juglone levels could be a result of the small sample size of the study or the failure of juglone to be affected within this range of edaphic variation. The Albany Pine Bush soil yielded higher extractable juglone than soil from the SUNY Experiment Station (Figure 3), suggest-

ing that juglone persistence in soil can be affected by greatly differing soil properties. Several studies suggest that juglone is less stable under alkaline conditions (Friedheim, 1934; Thomson, 1951; Marking, 1970), a situation that could partly explain the increased persistence of juglone in the more acidic Albany Pine Bush soil (Figure 3). Additionally, the lower organic matter content of the Albany Pine Bush soil also could be partly responsible for the increased extractable juglone, which is in agreement with a study by Dalton *et al.* (1983) with ferulic acid. In contrast, Ponder (1987) found soil pH to be lowest at a site that had the lowest soil juglone concentrations, and organic matter content to be positively correlated to soil juglone concentrations. How various soil properties, alone and in combination, affect soil juglone concentrations remains unclear.

The average chloroform-extractable soil juglone concentrations found during the fall beneath *J. nigra* was 1.88 μg/g soil (values corrected for recovery), which is similar to the average reported by Ponder and Tadros (1985) for a 14-year-old mixed *J. nigra, Elaeagnus umbellata* plantation in Illinois. Allelopathic effects were observed only in a mixed *J. nigra, Alnus glutinosa* plantation, where the soil juglone concentrations had reached 3.95 μg/g soil (Ponder and Tadros, 1985). These reported concentrations of chloroform-extracted juglone from field soil are within the range (10^{-5} M) shown to affect shoot elongation (Rietveld, 1981, 1983) and dry weight accumulation (Funk *et al.*, 1979; Rietveld, 1981, 1983) of various plant species in hydroponic systems, inhibit respiration of *Phaseolus* and *Lycopersicon* (Perry, 1967), and inhibit the growth of *Frankia* and *Rhizobium* (Dawson and Seymour, 1983). However, it is difficult to compare chloroform-extractable soil juglone concentrations with juglone solution concentrations.

Recovery Experiment. The recovery of commercial juglone added to soil *in vitro* drops substantially within a short period of time (<4 hr), suggesting that there must be a continuing input of juglone to soil in order for juglone to build up to detectable levels in the field (Figure 3). In contrast, Fisher (1978) found that juglone added to soil *in vitro* (50 ppm) remained toxic to *Pinus resinosa* seedlings for 30 days in dry soil and for 90 days in wet soil. This great difference in juglone persistence in soil between the two studies could be explained in part by differences in soil properties and/or differences in the amount of juglone initially added to soil. In this study, the juglone decline could not be fully attributed to microorganism degradation since juglone recovery from sterilized soil exhibited the same pattern (Figure 3). The greater juglone recovery with sterile soil compared to nonsterile soil could, in fact, reflect changes in the soil from the sterilization process and not necessarily the effects of microorganisms, although a common soil bacterium able to utilize juglone has been discovered (Rettenmaier *et al.*, 1983; Rettenmaier, 1985). It is suspected that abiotic processes must be largely responsible for this decline in extractable juglone. Whether juglone declines in the field at this high rate is unknown.

It is imperative to minimize the time between soil sampling and juglone extraction because chloroform-extractable juglone was found to decline quickly from soil. Actual juglone levels in soil were probably greatly underestimated in this study and that by Ponder and Tadros (1985), a result of the inability to analyze the soil samples immediately after collection. In addition, Ponder and Tadros (1985) oven-dried their samples for 24 hr prior to extraction, a process that could substanially reduce extractable juglone levels. When soil sample BW#6 was analyzed for juglone concentration before and after air drying for two days, the results showed substantial differences (De Scisciolo, 1988). The results of the recovery experiment on sample "100 dry" (Table 3), indicates that the reduction in extractable juglone upon soil drying is not due to differences in juglone extractability from soils of different moisture contents.

The extraction of juglone from soil using chloroform does not provide any information on the levels of juglone available to plants in the field. A major difficulty in allelopathy research is how to interpret the significance of laboratory results as they pertain to what actually occurs in the field. Although it is impossible to determine the amounts of juglone actually experienced by plants, the low recovery of juglone using water as the extracting solvent ($< 1\%$) suggests that concentrations of juglone in soil determined by chloroform extraction may not be experienced as such in the field.

Vegetation Analysis. Individual overstory trees can affect the understory physically (light quality and intensity, temperature, litter and organic matter accumulation, animal activity, etc.), as well as chemically (nutrient levels, allelochemical accumulation, pH, moisture, etc.). A spatial variation of soil properties develops in the understory depending upon tree species and distances away from individual tree bases (Voigt, 1960; Zinke, 1962; Gersper and Holowaychuk, 1970, 1971; Lodhi, 1977; Ponder and Tadros, 1985; Riha *et al.*, 1986; Crozier and Boerner, 1986). These patterns are site- and species-specific and are attributed to combined patterns of litter deposition (Zinke, 1962; Lodhi, 1977), stem flow (Voigt, 1960; Gersper and Holowaychuk, 1970, 1971; Crozier and Boerner, 1986), through-fall (Voigt, 1960; Tukey, 1966), and root exudation (Cook, 1921; Massey, 1925; Borner, 1960).

The localized areas beneath different tree species can be unique microhabitats for herbs and woody species. Numerous studies have found that overstory trees can explain some of the understory vegetational differences (Bratton, 1976; Hicks, 1980; Werner and Harbeck, 1982; Maguire and Forman, 1983; Everett *et al.*, 1983; Beatty, 1984; Crozier and Boerner, 1984; Turner and Franz, 1986). In contrast, some studies have found that overstory trees have a minor role in determining distributional patterns of understory vegetation (Collins *et al.*, 1984; Collins and Good, 1986). Site-specific factors can affect the degree of influence overstory trees have on the understory vegetation.

Allelopathy can be one cause of understory vegetational patterning. Many tree species have been suggested as being allelopathic (del Moral and Muller, 1970; Lodhi and Rice, 1971; Al-Naib and Rice, 1971; Lodhi, 1976; Chou and

Yang, 1982; Rice, 1984; Chou and Kuo, 1986). Although chloroform extraction yielded detectable levels of juglone in soil beneath *J. nigra* at the Experiment Station study site, *J. nigra* did not have any negative effect on the density and percent cover of the understory vegetation in comparison to the understory vegetation of *A. saccharum, Q. rubra,* and plots located away from the influence of tree bases (Table 4). The possibility exists that juglone, at least alone, might not be an allelochemical under field conditions. Alternatively, juglone concentrations might not have reached levels sufficient to negatively influence the understory vegetation. In addition, juglone could have been present in soil beneath the other tree species and control plots, which would obscure the results. Soil collected from an *A. saccharum, Q. rubra* stand (control stand) adjacent to site B did contain small amounts of juglone (De Scisciolo, 1988). Whether this juglone was actually present in soil or a contaminant from glassware is unknown. The roots of *J. nigra* are suspected as the major source of juglone in the soil (Cook, 1921; Massey, 1925; Schneiderhan, 1927; Pirone, 1938; Reinking, 1943; Strong, 1944; Sherman, 1971). *J. nigra* roots are extensive, widespreading (Holch, 1931; Biswell, 1935; Yen *et al.*, 1978), and concentrated in the upper 60 cm of soil (Yen *et al.*, 1978). Although soil juglone concentrations have been found to decrease with distance away from *J. nigra* (Ponder and Tadros, 1985), the distribution of juglone in soil throughout *J. nigra* stands is unknown.

J. nigra has a relatively open canopy, allowing greater light penetration to the understory. This canopy structure could explain why several understory species were positively associated with *J. nigra* (Table 5). The positive association between *Rubus occidentalis* and *J. nigra* has been previously documented (Brooks, 1951). *R. occidentalis* is an early successional species and requires high light levels. The negative association between *A. saccharum* seedlings and *J. nigra* is surprising since MacDaniels and Pinnow (1976) suggest that *Acer* spp. have a normal or improved growth in association with *J. nigra*.

The results of the ordinations revealed a strong separation of plots by site (Figures 4 and 5). The similarity between plots G and L are probably a result of the low dominance of *A. saccharum* at these two sites. In both the May and July ordinations, site B *J. nigra* represent a unique group. The similarity in the vegetation beneath *J. nigra* is most likely not a consequence of any strong allelopathic influences (Tables 4 and 5).

This study has demonstrated the presence of juglone in the soil beneath *J. nigra* in the field, but with no apparent allelopathic effect. Although there are claims of *J. nigra* allelopathy, no study has yet provided convincing evidence of *J. nigra* allelopathy according to criteria proposed by Fuerst and Putnam (1983) and Willis (1985). Even if *J. nigra* can have allelopathic effects, allelopathy may not occur at every site. Plant species can vary in their tolerance to juglone (Funk *et al.*, 1979; Rietveld, 1981, 1983). Similar-aged trees can vary from one another and from year to year in their production of allelochemicals

(Daglish, 1950; Graves *et al.*, 1979; Cline and Neely, 1984). Age, size, and density of *J. nigra* can be important factors in determining the accumulation of allelochemicals in soil (Rietveld, 1981). Lastly, variation in soil properties can affect the accumulation of allelochemicals in soil.

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SEX PHEROMONE OF *Manduca Sexta* (L) Stereoselective Synthesis of (10*E*,12*E*,14*Z*)-10,12,14-Hexadecatrienal and Isomers[1]

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Abstract—Three isomeric conjugated triene aldehydes, (10*E*,12*E*,14*Z*)-10,12-14-hexadecatrienal (IX), (10*E*,12*Z*,14*E*)-10,12,14-hexadecatrienal (XX), and (10*E*,12*E*,14*E*)-10,12,14-hexadecatrienal (X), two of which are components of the *Manduca sexta* (L.) sex pheromone, have been stereoselectively synthesized and spectroscopically characterized.

Key Words—Conjugated triene aldehydes, tobacco hornworm, Lepidoptera, Sphingidae, spectroscopic characterization, hexadecatrienal isomers.

INTRODUCTION

The isolation and identification of the female produced sex pheromone of the tobacco hornworm moth, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), was recently reported (Tumlinson et al., 1989) as a blend of saturated and mono-, di-, and triunsaturated aldehydes with chain lengths of 16 and 18 carbons obtained by rinsing the pheromone glands excised from calling *M. sexta* females with hexane. In a laboratory wind tunnel two components of this blend were required to stimulate *M. sexta* males to complete a characteristic behavioral sequence: upwind-oriented flight in the pheromone plume, approaching and touching the pheromone source, and bending their abdomens in apparent copulatory attempts. One of the two essential components was determined to be

[1] This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by USDA.

the previously identified (10*E*,12*Z*)-10,12-hexadecadienal (10*E*,12*Z*–16:Al, "bombykal")[2] (Starrat et al., 1979), while the other was identified as (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal (10*E*,12*E*,14*Z*–16:Al). Additionally, (10*E*,12*E*,14*E*)-10,12,14-hexadecatrienal (10*E*,12*E*,14*E*–16:Al) was found in the gland rinse. Although 10*E*,12*E*,14*E*–16:Al did not appear to be behaviorally active in wind-tunnel tests, it did stimulate one of two pheromone receptor cells in some of the trichoid sensilla on the antennae of *M. sexta* males (Kaissling et al., 1989). Initially, the isomeric configuration of the natural trienals was unknown, and their identity had to be confirmed by synthesis. Since the conjugated dienals produced by the insect are (10*E*,12*Z*)- and (10*E*,12*E*)-10,12-hexadecadienal, we concluded (Tumlinson, et al., 1989) that among the eight possible isomers of 10,12,14-hexadecatrienal, the *E*,*Z*,*E*; *E*,*E*,*Z*; and *E*,*E*,*E* were the most likely candidates for the unknown trienals. Herein we report the full experimental details of the syntheses of 10*E*,12*E*,14*Z*–16:AL, 10*E*,12*E*,14*E*–16:AL, and 10*E*,12*Z*,14*E*–16:Al with supporting spectral information.

METHODS AND MATERIALS

Solvents and Chemicals

The tetrahydrofuran (THF) used in all reactions was dried and freed from oxygen by distillation from benzophenone ketyl under nitrogen. Methylene chloride was dried by distillation from calcium hydride (CaH_2) under nitrogen. Hexamethylphosphoramide (HMPA) was dried over 13× molecular sieves. *N*,*N*-dimethylformamide (DMF) was distilled from CaH_2 at atmospheric pressure. The potassium carbonate (K_2CO_3) used for drying solutions of products, was anhydrous grade (Aldrich Chemical Co., catalog number 20,961-9).

Instrumentation

Infrared spectra (IR) were recorded with a Nicolet model 20 SXC FT-IR spectrometer. Compounds were analyzed either in 4% w/v solutions in CCl_4 (in cases where the products could be distilled or recrystallized) or as individual, vapor-phase, effluent peaks in the gas chromatographic–FT-IR mode (in cases where the products still contained traces of solvents or other impurities). In the latter case, the FT-IR was interfaced with a Hewlett-Packard 5890 gas chromatograph equipped with a 23-m × 0.32-mm-ID fused silica capillary column

[2] In the interest of consistency, since we have opted to use locants in the nomenclature (IUPAC, 1970) in this manuscript, we have deviated from the widely used and accepted shorthand for unsaturated functionalized, hydrocarbon type pheromones, i.e., 10*E*,12*Z*–16:Al would normally have been written *E*10,*Z*12–16:Al and so forth.

coated with 0.25 μm of methyl silicone. The ultraviolet spectra were recorded with a Varian model DMS 100 recording ultraviolet/visible spectrophotometer in absolute ethanol solutions.

^{1}H nuclear magnetic resonance (NMR) data were obtained with a Nicolet 300-MHz FT-NMR spectrometer on 1% solutions in $CDCl_3$. The spectrometer was interfaced to a Nicolet model 1280 data system collecting 16K data points (spectral width 3000 $\pm$ 1500 Hz) with a 9.75-μsec pulse equal to 90°. Chemical shifts are reported in parts per million downfield from tetramethylsilane as an internal standard.

Gas chromatographic–mass spectrometric (GC-MS) analyses were obtained on a Nermag model R1010 mass spectrometer using isobutane as the ionizing gas. The spectrometer was interfaced to a Hewlett-Packard model 5790 gas chromatograph equipped with both a split/splitless injector and a SGE OCI-3 cold, on-column injection system. Samples were analyzed with both injection systems. Helium was used as the carrier gas in all columns. In the splitless mode, injections were made with a 30-sec splitless delay. A 50-m × 0.32-mm-ID BPI (Scientific Glass Engineering, Inc.) fused silica column was operated at 80°C for the first 2 min and then temperature programmed at 32°/min to 230°C. A 50-m × 0.25-mm-ID CPS-1 (Quadrex corporation) fused silica column was operated at 50°C for 2 min and then temperature programmed at 10°/min to 180°C. In the on-column injection mode, the injector was maintained at room temperature during a 20-sec injection onto a 50-m × 0.25-mm-ID OV-1 fused silica capillary column. The column was held at 60°C for 1 min after injection and then temperature programmed at 15°/min to 170°C.

Gas chromatographic analyses were performed on Varian Aerograph models 1400, 2100, and 3700. All were fitted with user-designed, all-glass, capillary split-inlet systems with carrier gas (He) linear flow velocities of 18 cm/sec and a split ratio of 100:1. The columns (all fused silica) used were: 32 m × 0.32 mm ID, 0.25-μm film thickness CPS-1 (Quadrex Corporation); 15 m × 0.25 mm ID, 0.25-μm film thickness OV-1701 (Quadrex Corporation); 12 m × 0.22 mm ID, 0.25-μm film thickness BP-1 (Scientific Glass Engineering, Inc.). All products containing the conjugated triene system were analyzed on a Hewlett-Packard model 5890A gas chromatograph equipped with an on-column injection system. The column was a 30-m × 0.32-mm-ID, 0.25-μm film thickness, Supelcowax 10 (Supelco, Inc.). The oven temperature was held at 60°C for 1 min, then programmed to 190°C at 30°C/min.

The triene aldehydes were purified by preparative, reverse-phase, high-performance liquid chromatography (HPLC). The column used was 250 × 22.5 mm ID stainless steel packed with Adsorbosphere HSC18 (7 μm) (AllTech Associates). The MeOH–H_2O (83:17) mobile phase was delivered at a flow rate of 9.0 ml/min by a Laboratory Data Control Constametric II pump. A Kratos Spectroflow 757 variable wavelength ultraviolet absorbance detector set

at 267 nm was used for sample detection. The products were recovered from the MeOH—H_2O effluent by addition of an equal volume of water followed by small multiple extractions with chloroform. The organic extract was dried by passing it through anhydrous potassium carbonate under N_2 pressure. The methanol was GC^2 grade and the water HPLC grade from American Burdick and Jackson (B&J). The water was purified by freezing slowly and partial thawing, discarding that portion that thawed last. The chloroform used for recovering the triene aldehydes from the reverse-phase chromatographic effluent was GC^2 (B&J). Triene alcohols and aldehydes were also analyzed for isomer content on a 250 × 4.6-mm-ID stainless-steel HPLC column packed with 5 μm Adsorbosphere HSC18 (AllTech Associates) operated with a mobile phase of 80:20 MeOH—H_2O at 2 ml/min.

Reactions requiring dry oxygen-free conditions were conducted in glassware that had been dried at 130–140°C for 2 hr and cooled under a stream of nitrogen, and a positive nitrogen atmosphere was maintained during the reaction.

*Synthesis of (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal (IX) (Figure 1, scheme A)*

(*E*)-11-Iodo-10-undecen-1-ol (II) was prepared by the method of Miyaura et al. (1983). The product was vacuum distilled (bp 111–112°C/0.02 mm) (reported bp 100–110°C/0.1 mm; Miyaura et al., 1983) to give II in 43% yield. The IR and NMR data were in complete agreement with those of Miyaura.

*(*E*)-11-Iodo-10-undecen-1-ol* tert-*butyldimethylsilyl ether (III).* This silyl ether was prepared in quantitative yield by the method of Corey and Venkateswarlu (1972). IR (CCL_4) 3060 and 3010 cm^{-1} (HC=CH), 1097 cm^{-1} (C—O, Si—O), 945 cm^{-1} (E—HC=CH), 837 cm^{-1} (Si—C), 661 cm^{-1} (C—I); NMR δ 0.80 [s, 9H, (CH_3)$_3$CSi], 1.2 (s, 14H, CH_2-), 2.0 (dt,2H,CH_2HC=CH), 3.5 (t,2H,SiOCH_2-), 5.9 (d,1H,J = 16 Hz, HC=CHI), 6.5 (dt,1H, HC=CHI); CIMS (m/z) 411(M + 1)$^+$, 353(M + 1 − C_4H_{10})$^+$.

*(2*E*,4*E*)-14-Hydroxy-2,4-tetradecadienal* tert-*butyldimethylsilyl ether (IV).* Vinyl iodide III (12.3 g, 30 mmol), acrolein (16.8 g, 300 mmol), and palladium acetate (0.2 g 1.2 mmol) were added to a magnetically stirred suspension of finely pulverized potassium carbonate (passes a 0.0079-in. sieve) (9.8 g, 75 mmol) in DMF (100 ml). The mixture was cooled to 0°C and tetrabutyl ammonium chloride (8.4 g, 30 mmol) and additional DMF (100 ml, to facilitate stirring) were added. The mixture was stirred at 25°C for 3 hr. The product was isolated by pouring the reaction mixture into water (1000 ml) and hexane (300 ml) and stirring the resultant suspension for 1 hr. The grey solid was removed by filtration through Super-Cel. The layers were separated, and the aqueous layer was extracted (5 × 50 ml) with hexane–ether. The combined organic

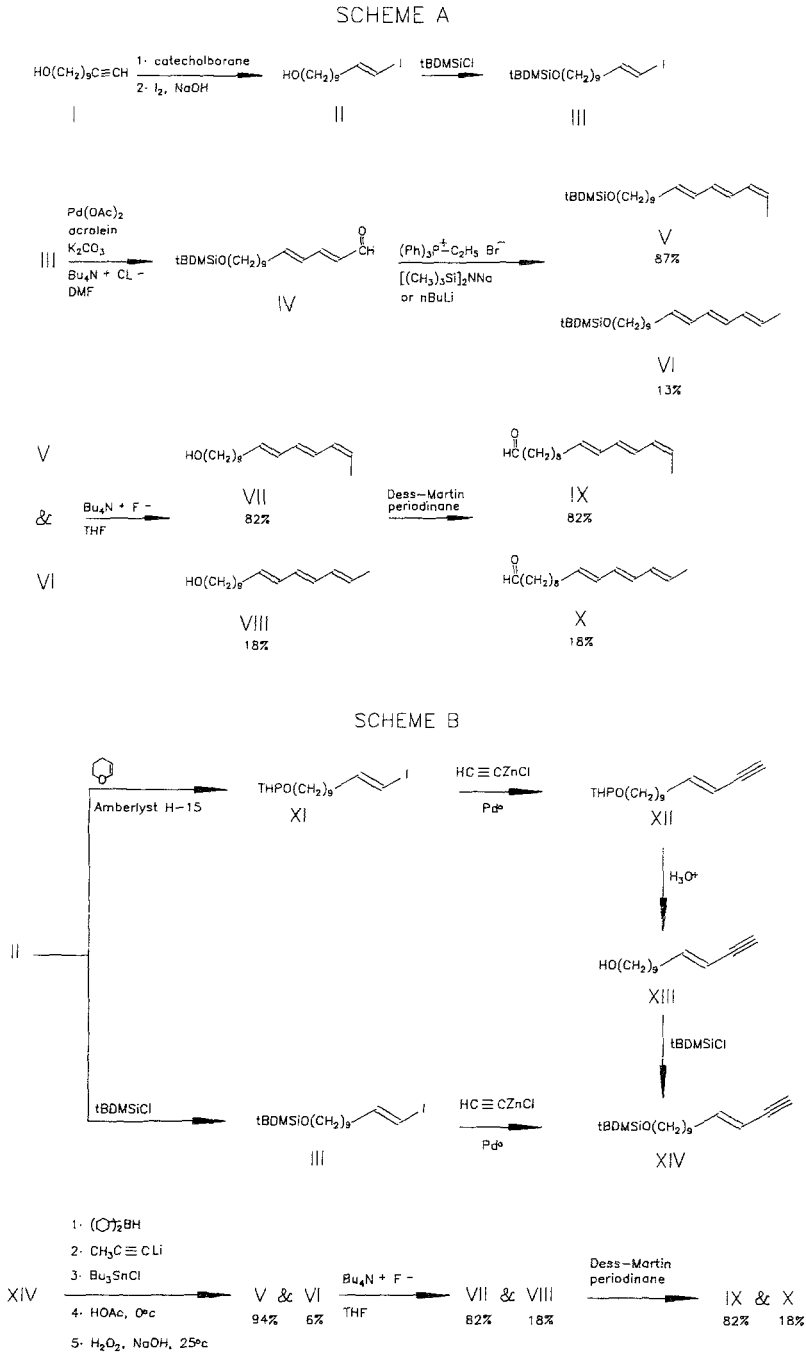

FIG. 1. Synthesis of (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal. tBDMSiCl = *tert*-butyldimethylchlorosilane.

extracts were washed with H_2O and satd. NaCl and dried over anhydrous K_2CO_3. Removal of the drying agent and evaporation of the solvents, gave IV as a yellow oil in 64% yield. IR (CCl_4) 3024 and 3008 cm^{-1} (HC=CH), 2734 and 2706 cm^{-1} (HC=O), 1689 cm^{-1} (C=O), 1674 cm^{-1} (HC=CH), 1109 and 1099 cm^{-1} (C—O, Si—O), 986 cm^{-1} (E—HC=CH), 837 cm^{-1} (Si—C); NMR δ ($CH_2CH_a{=}CH_bCH_c{=}CH_dCH_e{=}O$) 1.3 (s,14H,$CH_2$), 2.2 (dt,2H,$CH_2$HC=C), 3.55 (t,2H,$CH_2$OSi), 6.0–6.1 (dd,1H,$J_{cd}$ = 15.4 Hz, CH=CHCHO), 6.3(dt + dd,2H, CH_2CH=CH-CH), 7.0–7.1 (dd,1H,J_{bc} = 7.8 Hz, CHCH=CHCHO), 9.5 (d,1H,J_{de} = 8 Hz, CHO); CIMS (*m/z*) 339(M + 1)$^+$, 281(M + 1 − C_4H_{10})$^+$.

(10E,12E,14Z)-Hexadecatrien-1-ol tert-*butyldimethylsilyl ether (V).* Ethyl triphenylphosphonium bromide (7.0 g, 18.8 mmol) (Aldrich Chemical Co.) was placed in a dry flask under a N_2 atmosphere. HMPA (30 ml) and THF (75 ml) were added and the mixture cooled to ca. −15°C and sodium bis(trimethylsilyl)amide (18.8 ml, 18.8 mmol, 1.0 M solution in THF) (Aldrich Chemical Co.) was added slowly dropwise with stirring, keeping the reaction temperature < −10°C. When addition was complete (solution dark red), stirring was continued for 15 min at −15°C, then the temperature was lowered to −78°C, and aldehyde IV (5.1 g, 15 mmol), in THF (30 ml), was added dropwise. The reaction mixture was stirred at −78°C for 1 hr, the bath was removed, and the temperature brought to ambient. The volume of solvent in the reaction flask was reduced by one half by evaporation under aspirator vacuum with no application of heat, and then an equal volume of hexane was added. The mixture was stirred for 10 min, and poured into a separatory funnel. The layers were then separated, and the organic layer was washed with H_2O (3 × 25 ml) and satd. NaCl. The organic extract was dried by filtration through anhydrous K_2CO_3 under N_2 pressure. This organic extract was allowed to stand in a freezer (−30°C) overnight to precipitate the bulk of the triphenylphosphine oxide (Ph_3PO). This precipitate was removed by filtration through Super-Cel under N_2 pressure. Residual Ph_3PO was removed by passing (with N_2) a concentrated solution of the crude product through a 30-cm × 2-cm-ID flash chromatography column packed with silica gel modified with a bonded polar phase [bonded phase: diol (COHCOH), Cat. No. 7047, J.T. Baker Co., Phillipsburg, New Jersey] and eluting with hexane. The combined product containing fractions were concentrated without heat on a rotary evaporator to 250 ml and the total yield of trienes estimated by GC to be quantitative with the ratio of isomers V and VI being estimated (GC) at 87:13 *E,E,Z* to *E,E,E* IR (V) (vapor) 3027 cm^{-1} (HC=CH), 1107 cm^{-1} (Si—O), 988 cm^{-1} (conjugated E—HC=CH); NMR (V + VI) δ 0.9 (s,9H, $(CH_3)_3$CSi), 1.3 (s,14H,CH_2), 1.75 (d,3H,J = 6.9,CH_3HC=C), 2.0–2.1 (dt,2H,CH_2HC=C), 3.6 (t,2H,SiOCH_2), 5.4–5.5 (dq,1H,HC=CHCH$_3$), 5.6–5.8 (dt,1H,CH_2HC=C), 6.0–6.2 (m,3H,CH=CHCH=CHCH=CHCH$_3$), 6.4 (dd,1H,C=CHCH=CHCH$_3$); CIMS (V) (*m/z*) 351(M + 1)$^+$, 293(M + 1 − C_4H_{10})$^+$.

(10E,12E,14Z)-10,12,14-Hexadecatrien-1-ol (VII). The *tert*-butyldimethylsilyl ether (2.10 g, 6.0 mmol), as a concentrated solution in hexane (10 ml), was added to THF (150 ml) under N_2 and chilled to 5°C. Tetrabutylammonium fluoride (12 ml, 12 mmol, 1.0 M solution in THF) (Aldrich Chemical Co.) was added dropwise. The reaction was stirred for 3 hr at 25°C. The reaction was worked up by pouring it into cold H_2O (100 ml) and extracting the aqueous layer with 1:1 hexane–ether (3 × 50 ml). The organic extract was washed with H_2O (2 × 25 ml) and satd. NaCl. It was dried over anhydrous K_2CO_3 under refrigeration, then purified on the flash diol column in the same manner as V and VI. The total yield of trienes estimated by GC was 93%. This consisted of 82% VII and 18% VIII. This mixture of VII and VIII was crystallized from hexane at low temperatures (−30°C freezer) leaving most nonpolar impurities in the mother liquor. IR (VII) (vapor) 3027 cm^{-1} (HC=), 1048 cm^{-1} (C—O), 987 cm^{-1} (conjugated *E*—HC=CH); NMR (VII + VIII) δ 1.3 (s,14H,CH_2), 1.75 (d,3H,J = 7 Hz, C=CHCH_3, 2.0–2.1 (dt,2H, CH_2HC=C), 3.7 (t,2H,CH_2OH), 5.4–5.5 (dq,1H,HC=CHCH_3), 5.6–5.8 (dt,1H,CH_2HC=C), 6.0–6.2 (m,3H,olefinic), 6.4 (dd,1H,C=CHCH=CHCH_3) CIMS (VII) (*m/z*) 293(M + 57)$^+$, 279(M + 1 − 14)$^+$, 237(M + 1)$^+$, 223(M + 1 − 14)$^+$, 209(M + 1 − H_2O)$^+$.

(10E,12E,14Z)-10,12,14-Hexadecatrienal (IX). A solution of VII and VIII (0.56 g, 2.37 mmol) in hexane (10 ml) was added to a suspension of Dess-Martin periodinane (1.2 g, 2.6 mmol, Aldrich Chemical Co.) in methylene chloride (20 ml) under N_2 (Dess and Martin, 1983). The mixture was stirred at room temperature for 3 hr. Aliquots were removed periodically, worked up, and checked for reaction progress by GC analysis. Small amounts of the alcohol remained, so additional oxidant (0.7 g) was added, and the mixture was stirred an additional 2 hr, whereupon the reaction was complete. The reaction was worked up by adding diethyl ether (42 ml) and a solution of 0.5 M sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in saturated $NaHCO_3$ (42 ml) (this is a sevenfold excess of $S_2O_3^{-2}$ over oxidant). The mixture was vigorously stirred until the organic layer became clear (5 min), the layers were separated, and the aqueous layer was extracted with hexane–ether (1:1). The combined organic extracts were washed with H_2O (25 ml) and satd. NaCl solution. The extract was dried over anhydrous K_2CO_3, and the drying agent was removed by filtration under N_2 pressure. Most of the methylene chloride was removed by rotary evaporation, and the mixture of aldehydes was stored as a dilute solution in hexane–ether 1:1 at −30°C. The yield of trienals as estimated by GC was >80%, and the ratio of 10*E*,12*E*,14*Z*-16:Al IX to 10*E*,12*E*,14*E*-16:Al varied from 82:18 to 60:40. The isomeric aldehydes were separated by preparative HPLC, with the *E,E,Z* isomer eluting first. The pure products were recovered from the MeOH—H_2O effluent as described. IR (IX) (vapor) 3028 and 3019 cm^{-1} (HC=CH), 2864 and 2710 cm^{-1} (HC=O), 1741 cm^{-1} (HC=O), 990 cm^{-1} (*E*—HC=CH); NMR and CIMS, see Figures 3 and 4.

Synthesis of (10E,12E,14Z)-10,12,14-hexadecatrienal (IX) (Figure 1, scheme B)

(E)-11-Iodo-10-undecen-1-ol THP ether (XI). Iodoalcohol II (1.48 g, 59 mmol), Amberlyst 15 ion-exchange resin (1.48 g, 59 meq, Aldrich Chemical Co.) and 3,4-dihydro-2H-pyran (5.4 ml, 70.8 mmol, Aldrich Chemical Co.) were stirred together for 24 hr in dry hexane (20 ml) under a N_2 atmosphere. At the end of this time, analysis by GC, on an on-column injection system, and by FT-IR indicated that no alcohol remained. The catalyst was removed by filtration through Super Cel, and the solvent was evaporated. The yield of XI was quantitative. IR(CCl_4) 3060 and 3010 cm^{-1} (HC=CH), 1080 cm^{-1} (C—O), 940 cm^{-1} (*E*—HC=CH); NMR δ 2.05 (dt,2H, CH_2HC=CHI), 3.37, 3.49, 3.72, and 3.85 (4m,4H,CH_2O of chain and THP ring), 4.6 (2t,1H,OCHO), 5.9 (d,1H,*J* = 14.4 Hz, *E*—CH_2HC=CHI), 6.5 (dt,1H,*E*—CH_2HC=CHI); CIMS (*m/z*) 381(M + 1)$^+$, 253(M + 1 − HI)$^+$.

(E)-10-Tridecen-12-yn-1-ol (XII). Under a N_2 atmosphere, anhydrous $ZnCl_2$ (9.1 g, 66 mmol) (dried by heating under vacuum at 118°C for 12 hr) in THF (70 ml) was added dropwise to a stirred suspension of lithium acetylide ethylene diamine complex (6.8 g, 66 mmol, Aldrich Chemical Co.) in THF (60 ml) at 0°C. After addition, the suspension was stirred at 25°C for 2 hr, the temperature was lowered to 0°C, and the vinyl iodide XI (22.5 g, 59 mmol) in THF (20 ml) was added followed by tetrakis(triphenylphosphine) palladium (0) (3.45 g, 2.95 mmol, 5 mole % based on iodide) (Coulson et al., 1972; Colquhoun et al., 1984). The solid catalyst was added in portions from a small Erlenmeyer flask, with the aid of a piece of Gooch tubing. The mixture was stirred at 25°C for 16 hr and worked up by pouring into ice cold 1 N hydrochloric acid (200 ml) and pentane (100 ml). The suspension was stirred for 15 min and then filtered through Super Cel to remove the catalyst residues. The layers were separated, and the aqueous phase was extracted several times with pentane. The combined organic extracts were washed with water, satd. $NaHCO_3$, H_2O, satd. NaCl, and dried over anhydrous K_2CO_3.

Removal of the drying agent by filtration, evaporation of the solvents at 25°C, chromatography of the residual oil on silica gel (EM Science SX0143-3; 60–200 mesh) using hexane as eluant and gave a 57% yield of XII.

A portion of the crude product (8.4 g, 30 mmol) was hydrolyzed directly to (*E*)-10-tridecen-12-yn-1-ol XIII by stirring for 3 hr with a few drops of conc. HCl at ambient temperature in a mixture of H_2O (25 ml) and THF (100 ml). After the usual work-up an oil was obtained, and after recrystallization from 1:1 ether–pentane at −70°C, a heat-labile, air-sensitive, white solid, which melted at room temperature, was obtained in 43% overall yield from XI. IR(CCl_4) 3650 cm^{-1} (OH), 3320 cm^{-1} (≡C—H), 3050 cm^{-1} (HC=CH), 1050 cm^{-1} (C—O), 960 cm^{-1} (*E*—HC=CH); NMR δ 1.56

(m,4H,$\underline{CH_2}CH_2HC{=}CH$ and $\underline{CH_2}CH_2O$), 2.10 (dt,2H,$\underline{CH_2}CH{=}C$), 2.77 (s,1H,$\underline{HC}{=}C$), 3.64 (t,2H,$-\underline{CH_2}OH$), 5.45 (d,1H,J = 16 Hz, E - $HC{=}\underline{CHC}{\equiv}CH$), 6.24 (dt,1H,$\underline{HC}{=}CHC{\equiv}CH$); CIMS ($m/z$) 251$(M + 57)^+$, 195$(M + 1)^+$, 177$(M + 1 - H_2O)^+$.

(E)-10-Tridecen-12-yn-1-ol tert-*butyldimethylsilyl ether (XIV).* This compound was prepared in 96% yield from alcohol XIII by the same procedure as was used for the preparation of III from II. The product, which was labile to heat and air, was of sufficient purity to be used directly for the next step. IR (CCl_4) 3315 cm^{-1} ($\equiv\underline{C{-}H}$), 3035 cm^{-1} ($\underline{HC{=}CH}$), 1098 cm^{-1} ($\underline{C{-}O}$, $\underline{Si{-}O}$), 957 cm^{-1} ($E{-}\underline{HC{=}CH}$), 837 cm^{-1} ($\underline{Si{-}CH_3}$); NMR δ 0.0 (s,6H,Si$\underline{CH_3}$), 0.85 [s,9H, C($\underline{CH_3}$)$_3$], 1.25 [m,14H,($\underline{CH_2}$)$_7$], 2.08 (dt,2H,$\underline{CH_2}HC{=}C$), 2.75 (s,1H,$\underline{HC}{\equiv}$), 3.55 (t,2H,$\underline{CH_2}OSi$), 5.4 (d,1H,J = 16 Hz,$E{-}HC{=}\underline{CHC}{\equiv}CH$), 6.2 (dt,1H,$\underline{HC}{=}CHC{\equiv}CH$); CIMS ($m/z$) 365$(M + 57)^+$, 309$(M + 1)^+$, 293$(M + 1 - 14)^+$, 251$(M + 1 - 58)^+$.

The enyne *tert*-butyldimethylsilyl ether XIV was also prepared directly from iodide III in 86% yield by a modification of the procedure used to prepare XII from XI. The procedure was modified in that five equivalents of cold 2 M NH_4Cl solution were used in the place of 1 N HCl in the work-up. In addition, the crude dry product was passed through a short column of charcoal (coconut charcoal, Fisher 5-690-A), in lieu of silica gel or neutral alumina to remove the catalyst residues. This appeared to give a cleaner product as judged by GC analysis. The product was spectroscopically identical in all respects to that prepared from XIII.

(10E,12E,14Z)-Hexadecatrien-1-ol tert-*butyldimethylsilyl ether (V).* These reactions are carried out in two flasks. Dicyclohexylborane (15 mmol in 40 ml THF) was prepared in the first flask from borane–methyl sulfide complex and cyclohexene by the standard procedure (Brown, 1975). The enyne XIV (4.62 g, 15 mmol) in THF (12.5 ml) was added dropwise at 0°C to the dicyclohexylborane slurry, and the reaction mixture was stirred at 0°C for 2 hr, during which time the white slurry of dicyclohexylborane dissolved. The second flask was charged with THF (11 ml) chilled to −65°C, and propyne (2.55 ml, 45 mmol, Farchan Laboratories) was collected, via a Dewar trap, in a dropping funnel and added to the THF in the flask. Methyllithium (10.7 ml, 15 mmol, 1.4 M solution in ether, Aldrich Chemical Co.) was added slowly to the propyne solution. After addition was complete, the resultant white slurry was stirred at −23°C for 1 hr. The contents of the first flask were cannula transferred to the second flask with N_2 pressure, making sure the temperature of the second flask was held < -23°C. The mixture was stirred at 0°C for 1 hr then chilled to −23°C and redistilled tri-*n*-butyltin chloride (4.5 ml, 5.4 g, 16.5 mmol, Alfa Products) was added. The mixture was allowed to reach room temperature and stirred for 1 hr. The reaction was then chilled to −23°C, the glacial acetic acid (3.4 ml, 960 mmol) was added slowly, and the mixture was stirred at 0°C

overnight. With the reaction temperature held at 0°C, CH_3OH (24 ml) and 3 N sodium hydroxide (24 ml, 72 mmol) were added followed by the dropwise addition of 30% H_2O_2 (3.6 ml, 36 mmol), keeping the temperature below 10°C. When the hydrogen peroxide addition was complete, the reaction mixture was allowed to reach 25°C over 1 hr, then it was diluted with an equal volume of water and extracted (5 × 25 ml) with pentane. The combined pentane extracts were washed with water, satd. NaCl, and dried over anhydrous K_2CO_3 in the refrigerator. The drying agent was removed by filtration under N_2 pressure, the volume was carefully reduced to about 25 ml, and the crude product was passed through the same type of diol column described previously to purify V. The primary impurity present in the crude product (V) is bis(tri-*n*-butyltin)oxide. Repeated chromatography and careful fractionation on the diol column removed most of the tin-containing impurity. The estimated yield (GC) of triene silyl ether was 21% with a ratio of *E,E,Z* to *E,E,E* of 94 : 6.

The mixture of isomers were quantitatively cleaved to VII and VIII as previously described, and the ratio of VII to VIII was 82 : 18. Oxidation of this mixture of alcohols to the mixture of aldehydes IX and X was accomplished in the same manner with similar results as in the first synthesis of 10*E*,12*E*,14*Z*-16 : Al.

Synthesis of (10E,12Z,14E)-10,12,14-Hexadecatrienal (XX) (Figure 2)

10-Undecyn-1-ol THP ether (XV). This intermediate was prepared from 10-undecyn-1-ol and dihydropyran by the method of Bongini et al. (1979) (described in detail for XI) in 85% distilled yield (bp 125–135°C/0.02–0.06 mm). IR (CCl_4) 3320 cm^{-1} (≡CH); NMR δ 1.95 (t,1H,C≡CH), 2.17 (dt,2H,CH_2C≡CH), 3.37, 3.49, 3.72, 3.85 (4m,4H,CH_2O of THP and hydrocarbon chain), 4.57 (2t,2H,OCHO); CIMS (*m/z*): 253(M + 1)$^+$, 151(M + 1 − THPOH)$^+$.

12-Hydroxy-2-dodecyn-1-ol THP ether (XVI). THP ether XV was converted to propargylic alcohol THP ether XVI in 68% yield (bp 150–160°C/0.15 mm) by the method of Schaap et al. (1965). IR (CCl_4) 3640 cm^{-1} (OH), 2230 cm^{-1} (weak, unsymmetrical $RC{\equiv}CR_1$) 1080 cm^{-1} (C—O); NMR δ 2.2 (t,2H,CH_2C≡C), 3.37, 3.49, 3.72, 3.88 (4m,4H,CH_2O of THP and hydrocarbon chain), 4.24 (s,2H,C≡CCH_2OH), 4.58 (2t,2H, OCHO); CIMS (*m/z*) 283(M + 1)$^+$, 199(M + 1 − THPOH)$^+$.

(E)-2-Dodecen-1,12-diol (XVII). Lithium tetrahydroaluminate (3.0 g, 79 mmol, Alfa Products) was added to THF (130 ml) under a N_2 atmosphere. This mixture is chilled (ice bath), and a solution of THP ether XVI (8.36 g, 65 mmol) in THF (32 ml) is added dropwise. After addition is complete, the mixture is heated under reflux for 6 hr. The reaction was worked up by addition of dry ether (50 ml), chilling in an ice bath, cautious sequential addition of H_2O

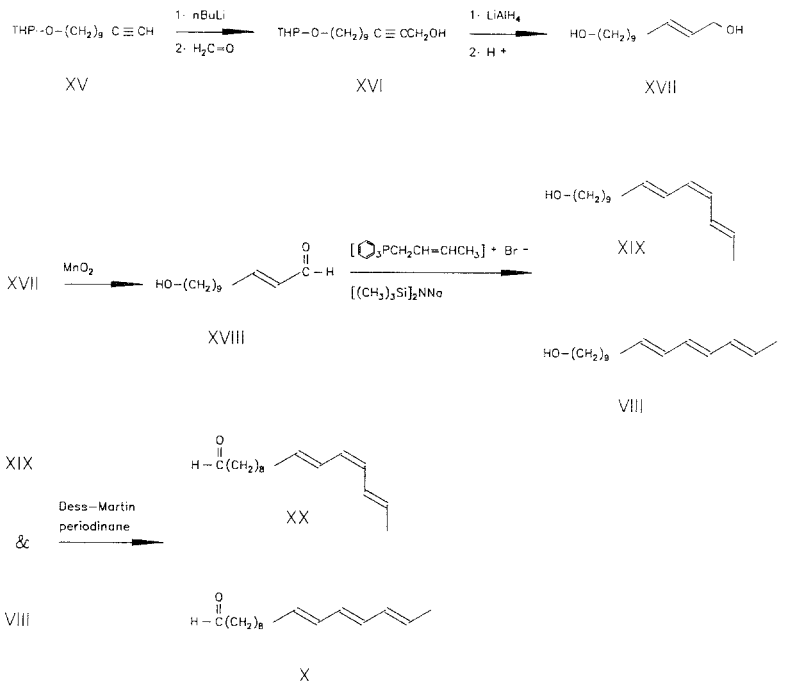

FIG. 2. Synthesis of (10*E*,12*Z*,14*E*)-10,12,14-hexadecatrienal.

(3.0 ml), 15% NaOH (3.0 ml), and H_2O (9.0 ml), and stirring at room temperature for 2 hr. The solid was removed by filtration through a fritted funnel and washed with anhydrous ether. The solvents were removed from the filtrate by rotary evaporation, and the crude product was cleaved to diol XVII by stirring the crude THP in CH_3OH at room temperature (8 hr) with Amberlyst catalyst (1.95 g) until GC analysis indicated that all the starting material was consumed. The catalyst was removed by filtration and the CH_3OH was evaporated. The residual oil was dissolved in a mixture of ether–hexane (3:2, 125 ml) and placed in a freezer (−30°C). The white solid that separted was collected on a cold funnel and recrystallized a second time from the same volume of ether–hexane to give 7.77 g (56%) of a white solid (mp 42–46°C). GC analysis indicated a purity of >95%. IR (CCl_4) 3350 and 3410 cm^{-1} (broad, OH), 1043 and 1056 cm^{-1} (C—O), 998 cm^{-1} (*E*—HC=CH); NMR δ 2.05 (dt,2H,CH_2HC=CH), 3.64 (t,2H,CH_2CH_2OH), 4.09 (d,2H,CH=CHCH_2OH), 5.65 (m,2H,HC=CH); CIMS (*m/z*) 183(M + 2 − H_2O)$^+$, 165(M + 2 − 2H_2O)$^+$.

*12-Hydroxy-(*E*)-2-dodecenal (XVIII).* The diol XVII (3.0 g, 15 mmol)

was stirred under N_2 at room temperature with MnO_2 (22.8 g, Aldrich Chemical Co., Cat. No. 21,764-6) in dry CH_2Cl_2 (150 ml). The oxidation was monitored by GC analysis on CPS-1 and was complete after 72 hr at room temperature. The MnO_2 was removed by filtration through a pad of Super Cel, and the solvent was evaporated. The residual oil was vacuum distilled (bp 125°C/0.05 mm) to yield 2.05 g (69%) of clear oil that was 99+ % pure by GC analysis on CPS-1. IR(CCl_4) 3420 cm^{-1} (broad OH), 2820 and 2740 cm^{-1} (HC=O), 1695 cm^{-1} (C=O), 1095 cm^{-1} (C—O), 983 cm^{-1} (*E*—HC=CH); NMR δ 2.35 (dt,2H,—CH_2HC=CH), 3.64 (t,2H,CH_2OH), 6.15 (dd,1H,C=CHC=O), 6.85 (dt,1H,C=CHCH_2), 9.5 (d,1H,HC=O); CIMS (m/e) 199$(M + 1)^+$, 181$(M + 1 - H_2O)^+$.

*(10*E*,12*Z*,14*E*)-10,12,14-Hexadecatrien-1-ol (XIX).* Crotyl triphenylphosphonium bromide (11.52 g, 29 mmol, mp 239–240.5°C, reported mp 245°C, Bohlmann and Mannhardt, 1956) was suspended in THF (60 ml) under a N_2 atmosphere. This mixture was chilled to −20°C, and *n*-butyllithium (12.4 ml, 29 mmol, 2.34 N solution in hexane, Alfa Products) was added dropwise. When addition was complete, the temperature of the mixture was raised to 0°C for 10 min and rechilled to −15°C. HMPA (30 ml) was added followed by aldehyde XVIII (2.6 g, 13 mmol) dissolved in THF (10 ml). The mixture was stirred at 0°C for 2 hr and then allowed to come to room temperature over 1–1.5 hr. It was worked up by pouring it into water (150 ml) and extracting the aqueous phase with 1:1 ether–hexane several times. The combined organic extracts were washed with water and satd. NaCl, dried over K_2CO_3, and stored in the refrigerator (+7°C). The drying agent was removed by filtration under N_2 pressure, and the volume of the filtrate was carefully (no heat, exclusion of air) reduced to 50 ml, and 50 ml of *n*-pentane was added. It was placed in the refrigerator overnight. The precipitated solid (Ph_3PO) was removed by filtration with N_2 pressure, and the filtrate was reduced to 50 ml and passed through a 12-cm × 15-mm-ID diol column using hexane as the eluant. Five fractions of 50 ml each were required to elute the triene alcohols from the column, but no Ph_3PO eluted. The yield was estimated by GC to be 82% with the ratio of XIX–VIII of 80:20. IR (XIX) (vapor) 3032 and 3003 cm^{-1} (HC=CH), 1050 cm^{-1} (C—O), 960 cm^{-1} (*E*—HC=CH); NMR (XIX + VIII) δ 1.55 (m,2H,CH_2OH), 1.79 (dd,3H,CH_3HC=CH), 2.10 (dt,2H,CH_2HC=CH), 3.64 (t,2H,CH_2OH), 5.70 (m,2H,*J* = 14.93 and 15.02 Hz, CH_3CH=C and CH_2CH=CH), 5.83 (2d,2H,*J* = 9.46 Hz,Z-CH=CH), 6.50 (m,2H,HC=CH); CIMS (XIX) (*m/z*) 293$(M + 57)^+$ 237$(M + 1)^+$, 223$(M + 1 - 14)^+$, 209$(M + 1 - 28)^+$.

*(10*E*,12*Z*,14*E*)-10,12,14-Hexadecatrienal (XX).* The alcohol mixture containing XIX and VIII was oxidized to a variable mixture of the aldehydes XX and X with Dess-Martin periodinane reagent as described for the 10*E*,12*E*,14*Z* isomer, and the isomers were separated by HPLC. IR (XX) (vapor) 3027 cm^{-1} (HC=CH), 2865 and 2711 cm^{-1} (HC=O), 1743 cm^{-1} (C=O); NMR and CIMS, see Figures 3 and 4.

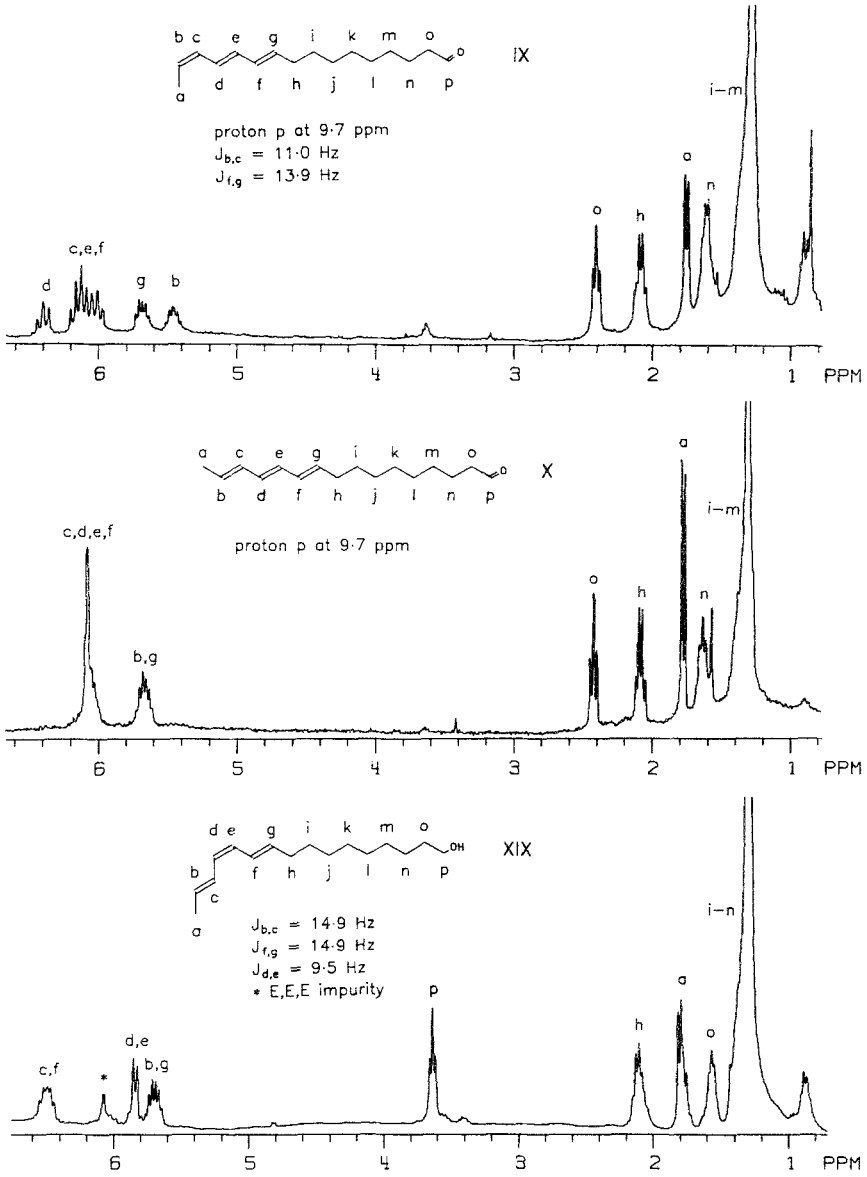

FIG. 3. Proton nuclear magnetic resonance spectra of (10*E*,12*E*,14*E*)- and (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal and (10*E*,12*Z*,14*E*)-10,12,14-hexadecatrien-1-ol.

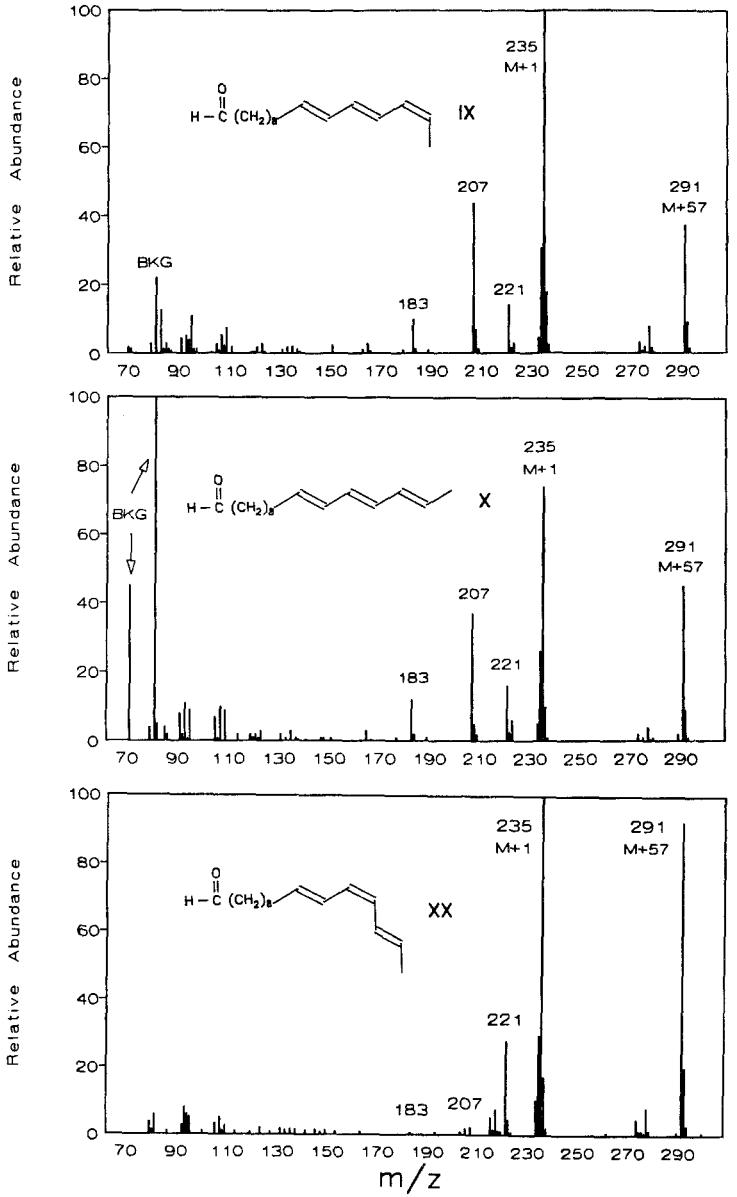

FIG. 4. Chemical ionization (isobutane) mass spectra of (10*E*,12*E*,14*E*)-, (10*E*,12*Z*,14*E*)-, and (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal.

(10E,12E,14E)-10,12,14-Hexadecatrien-1-ol (VIII). A dilute solution of XIX and VIII (100 mg/50 ml) in 1:1 hexane–ether was treated with I_2 (1–2 mg) and kept in laboratory light under N_2 for 10 min. The solution was washed with a few milliliters of saturated sodium thiosulfate solution and dried over K_2CO_3. Removal of the solvents and recrystallization of the residue from hexane–ether (90:10) gave pure VIII (mp 71–72.5°C). IR (VIII) (CCl_4) 3430 cm^{-1} (O—H), 1075 cm^{-1} (C—O), 995 cm^{-1} (*E*—HC=CH); NMR δ 1.56 (m,2H,CH_2CH_2OH), 1.76 (dd,3H,CH_3HC=CH), 2.08 (dt,2H,CH_2HC=CH), 3.63 (m,2H,CH_2OH), 5.64 (m,2H,HC=CH), 6.05 (m,4H,HC=CH). UV (absolute ethanol) 267 nm, ϵ = 42,192. CIMS (*m/z*) 293(M + 57)$^+$ 279(M + 1 − 14)$^+$ 237(M + 1)$^+$, 223(M + 1 − 14)$^+$, 209(M + 1 − 28)$^+$.

(10E,12E,14E)-10,12,14-Hexadecatrienal (X). The recrystallized alcohol VIII was oxidized to the corresponding aldehyde X with Dess-Martin periodinane as described for the other isomers. IR (vapor) 3018 cm^{-1} (HC=CH), 2865 and 2710 cm^{-1} (HC=O), 1744 cm^{-1} (HC=O), 989 cm^{-1} (*E*—HC=CH). NMR and CIMS, see Figs. 3 and 4.

Synthesis of Oxidation By-products (XXI, XXII, and XXIII) (Figure 5)

1,10-decanedial (XXI). 1,10-decanediol (Aldrich Chemical Co.) was oxidized in CH_2Cl_2 with pyridinium chlorochromate (Corey and Suggs, 1975) to XXI in 80% yield. IR (CCl_4) 2810 and 2710 cm^{-1} (HC=O), 1743 cm^{-1} (HC=O); NMR δ 1.31 (m,8H,CH_2), 1.61 (m,2H,CH_2CHO), 2.4 (m,2H,CH_2CH_2CHO), 9.76 (S,2H,CHO); CIMS (*m/z*) 171(M + 1)$^+$, 153(M + 1 − H_2O)$^+$, 135(M + 1 − $2H_2O$)$^+$.

(E)-2-Dodecene-1,12-dial (XXII). Diol XVII was oxidized to XXII with pyridinium chlorochromate in 90% yield. IR (vapor) 2805 and 2712 cm^{-1} (HC=O), 1742 and 1714 cm^{-1} (C=O); NMR δ 2.32 (dt,2H,CH_2CH=), 2.42 (dt,2H,CH_2CHO), 6.12 (dd,1H,HC=CHCHO), 6.85 (dt,1H,HC=CHCHO), 9.50 (d,1H,HC=CHCHO), 9.76 (s,1H,CHCHO); CIMS (*m/z*) 197(M + 1)$^+$, 179(M + 1 − H_2O)$^+$, 161(M + 1 − $2H_2O$)$^+$.

(2E,4E)-2,4-Tetradecadiene-1,14-dial (XXIII). Iodoalcohol II was converted to 14-hydroxy(*E,E*)-2,4-tetradecadienal by the same procedure used for the conversion of vinyl iodide III to protected dienal IV (Jeffery, 1985) in 40% yield. This intermediate was oxidized to XXIII in good yield with Dess-Martin periodinane. IR (CCl_4) 3021 cm^{-1} (HC=CH), 2812 and 2716 cm^{-1} (HC=O), 1730 cm^{-1} (CH_2HC=O), 1689 and 1643 cm^{-1} (HC=CHHC=CHCHO); NMR δ 2.21 (dt,2H,CH_2HC=CH), 2.42 (t,2H,CH_2CHO), 6.07 (dd,1H,*J* = 7.3 and 15.3 Hz, HC=CHCHO), 6.28 (dt,dd,2H,CH_2HC=CH), 7.08 (dd,1H,*J* = 7.3 and 11 Hz, HC=CHHC=CHCHO), 9.53 (d,1H,*J* = 7.96 Hz,C=CHCHO), 9.76 (s,1H,CH_2CHO); CIMS (*m/z*) 225(M + 1)$^+$, 223(M − 1)$^+$.

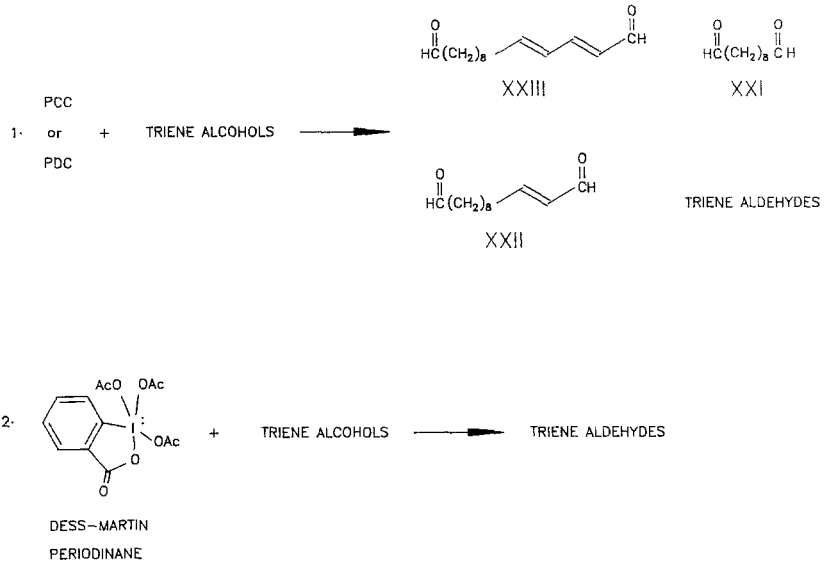

FIG. 5. Oxidation of isomeric trienols to aldehydes.

RESULTS AND DISCUSSION

Synthesis of (10E,12E,14Z)-10,12,14-Hexadecadienal (IX) (Figure 1, scheme A)

The synthesis of (10*E*,12*E*,14*Z*)-10,12,14-hexadecatrienal IX was accomplished by two different routes starting from a common starting material. These routes are presented in Figure 1. The first synthesis (Figure 1, scheme A) started with 10-undecyn-1-ol prepared from 10-undecen-1-ol (Givaudan Corp.) by the method of Miyaura et al. (1983) and proceeded through (*E*)-11-iodo-10-undecen-1-ol II. (*E*)-11-Iodo-10-undecen-1-ol *tert*-butyldimethylsilyl ether III was prepared from II with *tert*-butyldimethylchlorosilane. Since the GC purity of this intermediate was >90% and in light of the tendency of iodides to decompose upon distillation, III was utilized in the next step without further purification. The vinyl iodide III was converted to the α,β-ν,Δ unsaturated aldehyde IV in good yield using the method of preparing unsaturated carbonyl derivatives first described by Heck (1982) and subsequently modified by Jeffery (1985). Aldehyde IV was converted to a mixture of trienes V and VI with ethylidene triphenylphosphorane (Wittig reaction) under conditions [sodium bis(trimethylsilyl)amide as base] known to favor *Z* bond formation. This reaction was also run using *n*-butyllithium as the ylide-generating base with HMPA as a cosolvent (Sonnet, 1974) and gave the same ratio of isomers V and VI.

The use of ordinary silica gel, alumina, or Florisil for the chromatographic purification of triene products resulted in loss of product via decomposition. GC analyses of triene-containing products had to be conducted with cool, on-column injection, which was estimated to produce a 50% loss of product, since use of split injection systems resulted in even greater losses. Triene-containing products must be rigorously protected from oxygen and heat, therefore filtrations and evaporations of solvents must be done under N_2 at no higher than ambient temperatures.

The silyl ethers V and VI were cleaved to the free alcohols VII and VIII with tetrabutylammonium fluoride (Corey and Venkateswarlu, 1972) in virtually quantitative yield (GC estimation). The ratio of alcohols (*E,E,Z* to *E,E,E*) at this point was estimated to be 82 : 18. Apparently some isomerization of *E,E,Z* to *E,E,E* had taken place during the conversion of the silyl ethers to the alcohols. It was possible to obtain the IR and CIMS data for individual compounds from this mixture of alcohols because they are recorded from individual gas chromatographic effluent peaks. The [^{1}H]NMR spectrum of VII was obtained on a mixture of VII and VIII, but since the mixture was mostly (82%) VII, the spectrum was essentially that of VII. The mixture of VII and VIII was oxidized in 80% yield to the mixture of aldehydes (IX and X) with Dess-Martin reagent. The ratio of IX to X was variable, in some cases the same as the starting alcohols and in others a higher proportion of X was produced. This indicates that some isomerization of IX to X occurs during this oxidation.

The alternative synthetic route to IX (Figure 1, scheme B) also started with iodoalcohol II and proceeded through *tert*-butyldimethylsilyl ether III or THP ether XI. Initially, II was converted to (*E*)-11-iodo-10-undecen-1-ol THP. Since the crude product appeared to be of sufficient purity by GC analysis and because of the known tendency of tetrahydropyranyl ethers to decompose upon distillation, the crude product was used directly in the next step. We have determined that analyses of THP ethers in general are more reliable, as to the presence of starting alcohols, with on-column injection systems than with split injection systems.

Reaction of either THP ether XI or *tert*-butyldimethylsilyl ether III with ethynylzinc under Pd(O) catalysis (King et al., 1977, 1978) led to enynes XII and XIV. Initially we hydrolyzed crude enyne THP XII to enyne alcohol XIII because XIII proved to be an intermediate that could be readily purified by low-temperature (−70°C) crystallization and thus provided us with a pure intermediate to collect spectral data on. Subsequently, we were able to effect direct conversion of III to XIV, providing this *tert*-butyldimethylsilyl ether in excellent yield and sufficient purity to be used in the next step.

The enyne XIV was converted to triene V (together with 4% of its *E,E,E* isomer) in 21% yield by adaptation of a method for preparing diene hydrocarbons first reported by Zweifel and Backlund (1978) and applied to the synthesis

of functionalized dienes by Doolittle and Solomon (1986). An important modification of the original procedure (Zweifel and Backlund, 1978) was found to be critical in the present case. It was found that the cleavage of the carbon–boron and carbon–tin bonds (with glacial acetic acid) occurred readily at 0°C and, in fact, when this step was conducted at 50°C, as initially described, very little triene product was isolated. This result is a manifestation of the extreme sensitivity of the conjugated triene system to electrophilic attack. The crude product mixture was chromatographed, as in the case of synthesis A, and cleaved to the triene alcohol mixture VII and VIII in 65% yield. The ratio of VII to VIII at this point was 82 : 18. The mixture of alcohols was oxidized to the mixture of aldehydes IX and X with Dess-Martin reagent.

The overall yield was higher from synthesis 1, but the final product consisted of a mixture of the *E,E,Z* and *E,E,E* isomers. This route had the advantage of easier removal of by-products from the *tert*-butyldimethylsilyl ethers and alcohols. In the case of the second synthetic scheme, the stereospecificity appeared to be better, i.e., less *E,E,E* isomer was produced initially, but the removal of a by-product of the reaction, bis(tri-*n*-butyltin) oxide, proved to be difficult because of coelution with the silyl ethers from the diol column. Normally, this is removed by chromatography on florisil (Zweifel and Backlund, 1978), or silica-gel (Doolittle and Solomon, 1986), but these adsorbents destroyed and isomerized the *E,E,Z* isomer.

*Synthesis of (10*E*,12*Z*,14*E*)-10,12-14-Hexadecatrienal (XX) (Figure 2)*

Acetylenic alcohol I was converted to its THP ether XV and was chain-extended to the propargylic alcohol XVI with *n*-butyllithium and paraformaldehyde. Reduction of XVI (Molloy and Hauser, 1968), followed by hydrolysis of the crude product, produced diol XVII, which was a conveniently purified (solid, recrystallized from ether–hexane) intermediate. Diol XVII was regioselectively oxidized in good yield to the hydroxyaldehyde XVIII with manganese dioxide (Attenburrow et al., 1952; Fatiadi, 1976). This hydroxyaldehyde was utilized in a Wittig reaction, in a mixture of THF–HMPA, with two equivalents of crotyltriphenylphosphorane, producing the mixture of alcohols XIX and VIII. It proved easier to use two equivalents of phosphorane for the Wittig condensation than protecting the hydroxyl group of XVIII, conducting the Wittig reaction with one equivalent of phosphorane, and removing the protecting group. This sequence would have necessitated subjecting the trienes to one additional manipulation with its attendant risk for isomerization and O_2-catalyzed polymerization. The Ph_3PO was quite easily removed from the mixture of alcohols XIX and VIII by a combination of crystallization from hexane and chromatography on the diol column. When this reaction was run using sodium bis(trimethylsilyl)amide to generate the phosphorane with only THF as solvent,

the yield was 93% with a ratio of isomers of 85 : 15 (XIX–VIII). HPLC analysis on the reverse-phase analytical column (Figure 6) indicated the presence of a trace amount of VII. The spectral data on alcohol XIX was obtained in the same manner as that for VII in synthesis A. The mixture of alcohols VIII and XIX could be readily isomerized to a mixture rich in VIII. Pure VIII could be obtained from this mixture by crystallization and recrystallization, thus allowing the recording of its ultraviolet spectrum, which was in good agreement with those reported for other trienes (Crombie and Jacklin, 1957; Hopkins, 1972; Sonnet, 1968). It was not possible to obtain ultraviolet spectra on the other isomeric alcohols or aldehydes because the alcohols were never obtained completely pure and it was not possible to prepare a solution containing a precise amount of any of the aldehydes for ultraviolet analysis. The mixture of alcohols XIX and VIII was oxidized to the mixture of aldehydes XX and X with Dess-Martin's periodinane in good yield. However, the lability of 10*E*,12*Z*,14*E*-16 : Al was such that it was not possible to obtain a sample of sufficient purity to obtain a [^{1}H]NMR spectrum, although the IR and CIMS spectra could be recorded on pure chromatographic effluent peaks. The [^{1}H]NMR spectra and CI mass spectra of the isomeric aldehydes and alcohol XIX are presented in Figures 3 and 4.

The sensitivity of the conjugated triene to electrophilic attack, heat, and air has been reported (Crombie and Jacklin, 1957) and is manifested in the

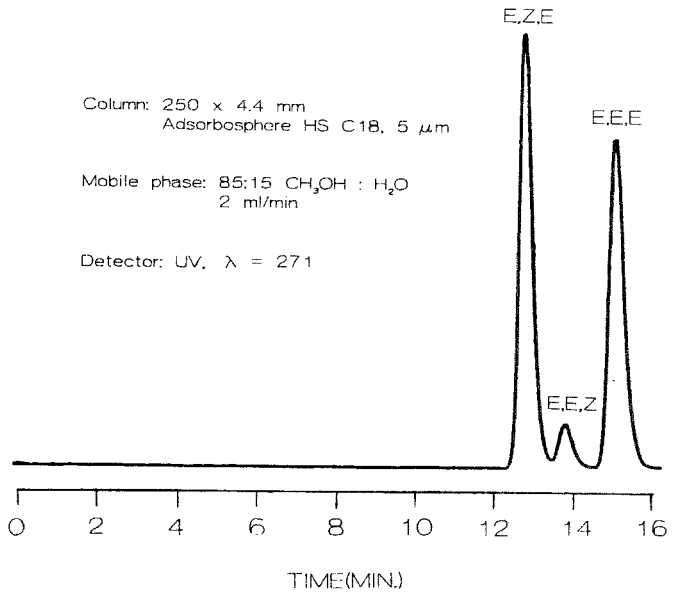

FIG. 6. High performance reverse phase chromatography of the isomeric aldehydes.

sensitivity to acid and electrophilic chromatographic adsorbents, gas chromatographic behavior, and the difficulty encountered in oxidizing the triene alcohols to aldehydes. First, in both syntheses, 1 and 2, the choice of protecting groups is critical. It was found that the removal of the *tert*-butyldimethylsilyl protecting group under basic conditions ($Bu_4N^+F^-$) was facile and gave good yields. The removal of protecting groups requiring acidic catalysis, e.g., THP ether, resulted in extensive isomerization and/or degradation. Also, in the course of most syntheses, the removal of impurities such as Ph_3PO by normal chromatographic procedures (silica gel, alumina, Florisil) would be trivial, but in light of the instability of these trienes towards those adsorbents, chromatography proved to be a potential stumbling block. Second, all yields of reactions after the conjugated triene structure was in place had to be estimated by GC quantification. Gas chromatographic analysis on the on-column injection system typically resulted in a 50% loss of material (this is factored into each yield estimate based on GC quantitation), and the use of systems with a split inlet system gave higher but unmeasured losses. Even with on-column injection, the peaks from chromatography of the *E,Z,E* isomer were always very broad and appeared typical of those where extensive isomerization–decomposition was occurring. Third, the use of the well-known oxidizing agents such as pyridinium chlorochromate (Corey and Suggs, 1975) and pyridinium dichromate (Corey and Schmidt, 1979) resulted in an unprecedented attack on the conjugated triene system producing the by-products shown in Figure 5. These by-products were identified by comparison of their retention times on several columns and their CIMS with those of authentic samples prepared by standard synthetic techniques. The two chromium oxidizing agents would effect the conversion of the triene alcohols to the corresponding aldehydes, but the amounts of the by-products XXI, XXII, and XXIII became excessive before conversion of the alcohol to aldehyde was complete. The use of other electrophilic oxidizing agents such as *N*-chlorosuccinimide/methylsulfide (Corey and Kim, 1972) resulted in very low yields and extensive isomerization. Thus far, we have only been able to successfully effect this conversion without extensive cleavage of the conjugated triene system with Dess-Martin's periodinane reagent, even though it does cause some variable and thus far uncontrollable isomerization.

The [^{1}H]NMR spectra of two of the isomeric aldehydes are presented in Figure 3 along with the spectrum of the *E,Z,E* alcohol. The olefinic protons of 10*E*,12*E*,14*Z*–16 : Al occur as a group of four signals between 5.2 and 6.5 ppm, whereas those for the *E,Z,E* isomer occur as a set of three signals between 5.6 and 6.6 ppm and those for the *E,E,E* isomer occur as two sets of signals between 5.5 and 6.2 ppm. Specific partial assignments were made through a series of spin-decoupling experiments. Only the *E,E,Z* and *E,Z,E* isomers were amenable to analysis using single irradiation techniques, but the assignments are in good agreement with the complete analysis (using double irradiation tech-

niques) of the methyl (9*Z*,11*E*,13*E*)-9,11,13-octadecatrienoate and (9*Z*,11*E*,13*Z*)-9,11,13-octadecatrienoate published by Ratovelomonana and Linstrumelle (1984) and that of the eight isomers of 10,12,14-hexadecatrienyl acetate reported by Ando et al. (1988) using double-irradiation techniques.

The CI mass spectra of all three aldehydes, using isobutane as the ionizing gas, are presented in Figure 4 and show some parallels with those produced by functionalized conjugated dienes with isobutane (Doolittle et al., 1985; Einhorn et al., 1985, 1987; Budzikiewicz et al., 1987). Specifically, the ion of *m/z* of 183 is strong evidence for the location of the first double bond in the conjugated system being between carbons 10 and 11. Functionalized conjugated dienes such as bombykal [(10*E*,12*Z*)-10,12-hexadecadienal] have a strong ion at *m/z* 183.

CONCLUSION

The three isomeric aldehydes 10*E*,12*E*,14*Z*-16:Al, 10*E*,12*E*,14*E*-16:Al, and 10*E*,12*Z*,14*E*-16:Al were stereoselectively synthesized and spectroscopically characterized. Spectroscopic and chromatographic data of the synthetic material were compared to data of the biologically active triene aldehyde and its *E*,*E*,*E* isomer isolated from the gland dip of *M. sexta* (L.) (Tumlinson et al., 1989). The synthesis of 10*E*,12*E*,14*Z*-16:Al reported herein corroborates the identification of this chemical as the second essential component of the pheromone blend of *M. sexta* (L.) and the synthesis of 10*E*,12*E*,14*E*-16:Al corroborates its presence in the gland dip.

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INHIBITORY PHEROMONAL ACTIVITY PROMOTED BY SULFUR ANALOGS OF THE SEX PHEROMONE OF THE FEMALE PROCESSIONARY MOTH *Thaumetopoea pityocampa* (DENIS AND SCHIFF)

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Abstract—New sulfur analogs of the sex pheromone of the female processionary moth *Thaumetopoea pityocampa* have been found to be effective inhibitors of the natural pheromone activity both in EAG bioassays and field tests. The structures of these analogs have been derived from replacement of the oxygen atom(s) of the acetate group by sulfur (compounds **3–5**) and the olefinic moiety of the enyne function by the isosteric SCH_2 group (compounds **6** and **7**). The synthesis and biological activity of 3-[(*Z*)-12-pentadecen-10-ynylthio]-1,1,1-trifluoropropan-2-one (**8**), a closely related structure to the pheromone is also described.

Key Words—Sex pheromone, inhibition, processionary moth, *Thaumetopoea pityocampa*, sulfur analogs, Lepidoptera, Thaumetopoeidae.

INTRODUCTION

The inhibition process of insect olfaction can be approached by irreversible activation of the receptors (hyperagonism) or by blockage of the pheromone recognition by a receptor cell (antagonism) (Prestwich, 1987). In this context, the design of pheromone analogs may lead not only to a better understanding of the binding interactions between the natural pheromone and the receptor proteins, but also to the development of new compounds potentially useful in pest control (Albans et al., 1984; Roelofs and Comeau, 1971; Camps et al., 1988). These analogs have been logically designed either by isosteric replacement of atoms and functions or by sterical modifications of the parent molecule with

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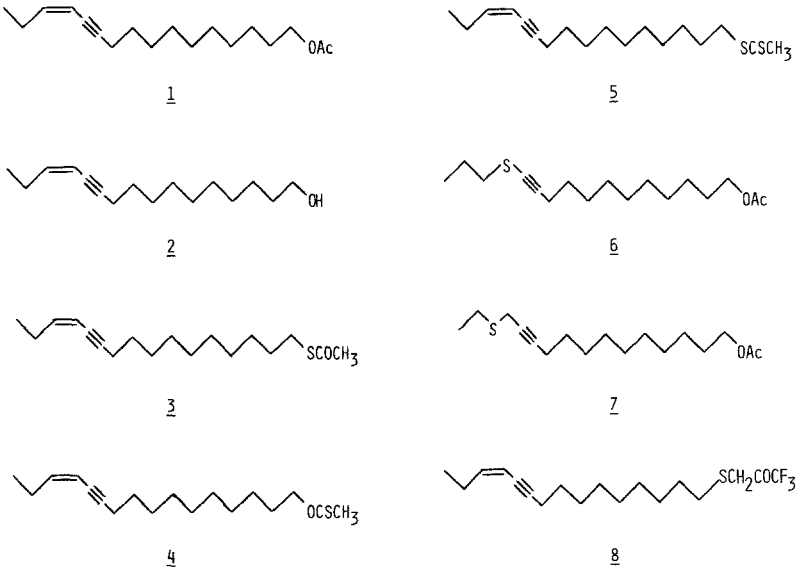
preservation of the original electronic environment (Priesner, 1979; Liljefors et al., 1985; Subchev et al., 1987).

The only component found so far in the female sex pheromone gland of the processionary moth *Thaumetopoea pityocampa* has been identified as (Z)-13-hexadecen-11-ynyl acetate **1** (Guerrero et al., 1981). Several efficient syntheses of this compound have been reported (Camps et al., 1981, 1983; Michelot et al., 1982; Shani et al., 1983; Cardillo et al., 1982; Stille and Simpson, 1987) and its attractant activity confirmed in field tests (Cuevas et al., 1983; Einhorn et al., 1983).

Continuing our efforts in the development of new analogs with synergistic or inhibitory activity of the sex pheromone of the processionary moth (Camps et al., 1986, 1988), we describe herein the synthesis and biological activity, in EAG bioassays and field tests, of sulfur analogs **3–8.** These compounds formally proceed from structural modifications on two of the three putative active sites of the parent molecule **1,** which could be involved in the interaction process with the antennal receptors, namely, the acetate group and the double bond of the enyne functionality. However, we have preserved the acetylenic moiety at C-11 since its presence has been shown to be essential to elicit a notable pheromonal activity (Camps et al., 1988). Thioesters **3** and **4** and dithioester **5** have resulted from replacement of the oxygen atom(s) in the acetate group by sulfur, whereas sulfides **6** and **7** proceed from isosteric replacement of the olefinic carbons by the CH_2S group (Scheme 1).



SCHEME 1.

On the other hand, in view of the reported inhibitory activity of insect juvenile hormone esterases by trifluoromethylketone derivatives (Hammock et al., 1982), we included 3-[(*Z*)-12-pentadecen-10-ynylthio]-1,1,1-trifluoropropan-2-one (**8**) in our pheromonal inhibition studies.

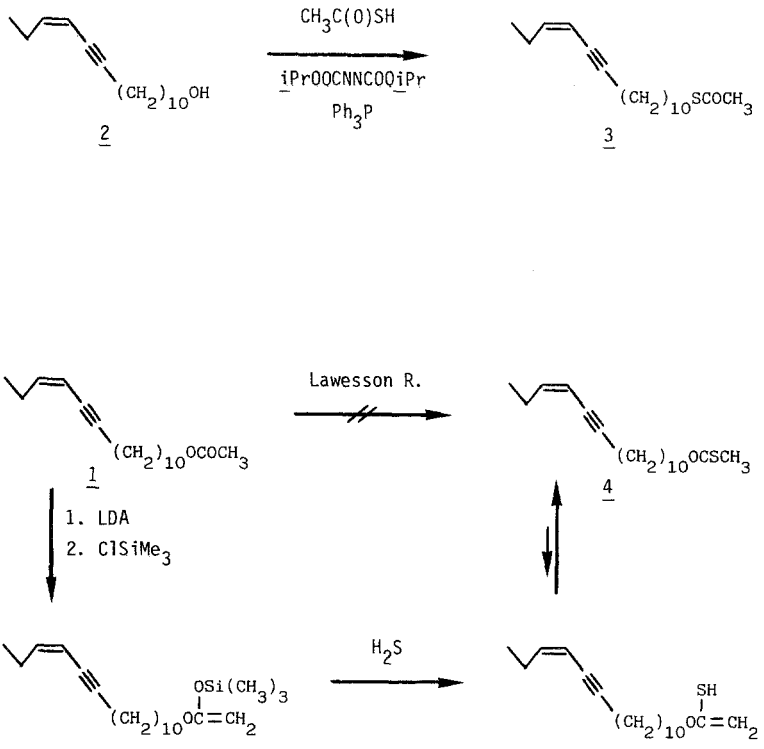
METHODS AND MATERIALS

Boiling points are uncorrected. Elemental analyses were determined on a Carlo Erba model 1106. IR spectra were recorded in CCl_4 solution on a Perkin Elmer 399B grating spectrometer. [^{1}H]- and [^{13}C]NMR spectra were determined in $CDCl_3$ solution on a Bruker WP80SY spectrometer, operating at 80 and 20.15 MHz, respectively, and absorptions are expressed in δ scale relative to TMS. [^{19}F]NMR spectra were recorded in $CDCl_3$ solution on the same instrument at 75.39 MHz, and the values are expressed in δ scale relative to trifluoroacetic acid (1% in $CDCl_3$) as external reference. GLC analyses were performed on Carlo Erba models 2350 and 4130, equipped with a FID detector, by using a 3% OV-101 glass column 2 m × 3 mm ID on Chromosorb W (nitrogen as carrier gas), or a fused silica capillary column SE-54 50 m × 0.32 mm ID (hydrogen as carrier gas). EI mass spectra were determined on a Hewlett Packard 5995C model using a OV-101 25-m × 0.25-mm-ID fused silica capillary column.

Reactions requiring anhydrous and oxygen-free conditions were performed under inert atmosphere (N_2 or A). Commercial reagents were from Fluka AG Buchs, (Switzerland) or Aldrich Chemie 1, Steinheim, (West Germany) and were used without further purification. Anhydrous solvents were prepared as follows: tetrahydrofuran (THF) by distillation from Na benzophenone, diethyl ether from lithium aluminum hydride (LAH), methylene chloride from P_2O_5, pyridine and diisopropylamine from KOH, and benzene and hexamethylphosphoric triamide (HMPT) from CaH_2.

S-(Z)-*13-Hexadecen-11-ynyl Thioacetate (**3**).* This compound was prepared by a method similar to that described by Volante (1981). Thus, to a solution of 1.15 g (4.40 mmol) of triphenylphosphine in 17 ml of anhydrous THF, previously cooled to 0°C, was added 0.94 g (4.64 mmol) of diisopropyl azodicarboxylate and the mixture stirred for 1 hr at the same temperature. Then, a solution of 0.53 g (2.25 mmol) of alcohol **2** and 0.366 g (4.81 mmol) of thioacetic S-acid in 9 ml of anh. THF was added and the mixture stirred at room temperature for 3 hr. The resulting solid was filtered and washed with ether. The combined organic phases were concentrated under reduced pressure to leave a residue, which was purified by column chromatography on silica gel eluting with hexane–ether 4 : 1 to yield 0.52 g (89%) of thioester **3** (Scheme 2).

Anal.: Calcd. for $C_{18}H_{30}OS$: C, 73.41; H, 10.27; S, 10.89. Found: C, 73.40; H, 10.37; S, 10.86. IR: ν 3010, 2920, 1690, 1135, 1105, 730 cm^{-1}.



SCHEME 2.

[^{1}H]NMR: δ 5.75 (dt, 1H, J = 10.5 and J = 7 Hz, $CH_2\underline{CH}{=}CH$), 5.40 (d, 1H, J = 10.5 Hz, $CH_2CH{=}C\underline{H}$), 2.50 (t, 2H, J = 6.5 Hz, CH_2SCO), 2.30 (c, 4H, $C\underline{H}_2CH{=}CHC{\equiv}CC\underline{H}_2$), 2.05 (s, 3H, $SCOCH_3$), 1.4 (b, 16H, $8CH_2$), 1.0 (t, 3H, J = 7.0 Hz, CH_3). [^{13}C]NMR: δ 195.7 (CO), 143.8 (C-14), 108.8 (C-13), 94.4 (C-11), 78.8 (C-12), 75.4 (C-1), 30.4 (C-1′), 28.2–24.4 (C-2 to C-9), 23.3 (C-15), 19.7 (C-10), 13.5 (C-16). MS (EI) *m/z* (relative intensity): 294 (M^+, 10), 265 (10), 251 (98), 183 (14), 135 (11), 161 (16), 107 (18), 93 (48), 79 (82), 67 (15), 55 (18).

O-(Z)-*13-Hexadecen-11-ynyl Thioacetate* (**4**). To a solution of 0.17 ml (1.19 mmol) of diisopropylamine in 2 ml of anh. THF, cooled to 0°C and under A, was slowly added 1.25 ml of a 0.95 M BuLi (1.19 mmol) in hexane. The mixture was stirred at this temperature for 30 min and cooled to −78°C. Then a mixture of 300 mg (1.079 mmol) of acetate **1** in 5 ml of anh. THF and 140 mg (1.28 mmol) of trimethylsilyl chloride were added and the mixture stirred at that temperature during 10 min (Corey and Wrigth, 1984). After this period of time, the mixture was warmed to −10°C and a stream of dry H_2S was bub-

bled through the solution during 15 min, warmed to room temperature and further stirred for 2 hr. The solvent was stripped off and the residue was chromatographed on silica gel eluting with hexane–ether 5 : 1 to obtain 215 mg (69%) of thioester **4** (Scheme 2).

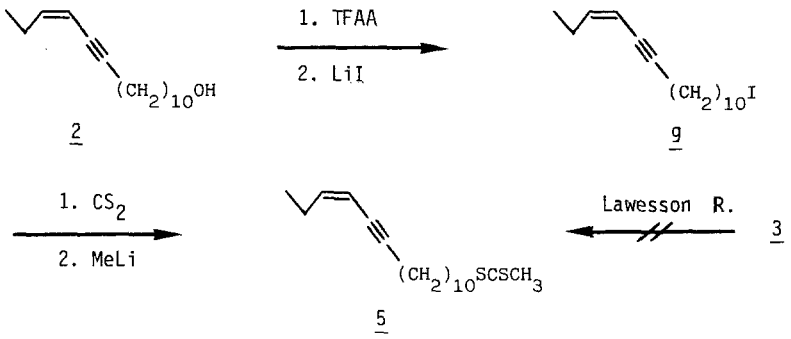
Anal. Calcd. for $C_{18}H_{30}OS$: C, 73.41; H, 10.27; S, 10.89. Found: C, 73.28; H, 10.37; S, 10.87. IR: ν 3010, 2930, 1600, 1240, 640 cm^{-1}. [^{1}H]NMR: δ 5.8 (dt, 1H, J = 10.5 Hz and J = 7 Hz, $CH_2C\underline{H}{=}CH$), 5.45 (d, 1H, J = 10.5 Hz, $CH_2CH{=}C\underline{H}$), 3.4 (t, 2H, J = 7 Hz, CH_2OCS), 2.4 (s, 3H, $OCSCH_3$), 2.25 (c, 4H, $C\underline{H}_2CH{=}CHC{\equiv}CC\underline{H}_2$), 1.4 (b, 16H, $8CH_2$), 0.97 (t, 3H, J = 7 Hz, CH_3). [^{13}C]NMR: δ 219.0 (CS), 143.9 (C-14), 108.5 (C-13), 94.5 (C-11), 78.7 (C-12), 64.0 (C-1), 34.5 (C-1′), 28.5-23.4 (C-2 to C-9 and C-15), 19.5 (C-10), 13.4 (C-16). MS (EI) *m/z* (relative intensity): 294 (M^+, 19), 279 (11), 251 (43), 161 (5), 154 (16), 147 (11), 139 (4), 133 (22), 121 (20), 111 (20), 105 (38), 94 (79), 79 (100), 67 (46), 55 (65).

*1-Iodo-(Z)-13-hexadecen-11-yne (**9**).* This compound was prepared according to a procedure previously described by us (Camps et al., 1987). Thus, starting from 313 mg (0.904 mmol) of alcohol, 227 mg (1.08 mmol) of trifluoroacetic anhydride and 268 mg (4.52 mmol) of anhydrous lithium iodide, 244 mg (78%) of compound **9** was obtained.

Anal.: Calcd. for $C_{16}H_{27}I$: C, 55.33; H, 7.78. Found: C, 55.28; H, 7.73. IR: ν 3020, 2920, 2850, 1610, 1460, 740 cm^{-1}. [^{1}H]NMR: δ 5.85 (dt, 1H, J = 10.5 Hz and J = 7 Hz, $CH_2C\underline{H}{=}CH$), 5.4 (d, 1H, J = 10.5 Hz, $CH_2CH{=}C\underline{H}$), 3.18 (t, 2H, J = 6.3 Hz, CH_2I), 2.3 (c, 4H, $C\underline{H}_2CH{=}CHC{\equiv}CC\underline{H}_2$), 1.7 (c, 2H, $C\underline{H}_2CH_2I$), 1.3 (b, 14H, $7CH_2$), 0.93 (t, 3H, J = Hz, CH_3). MS (EI) *m/z* (relative intensity): 346 (M^+, 100), 317 (52), 303 (13), 154 (10), 149 (16), 135 (47), 121 (26), 107 (25), 95 (30), 79 (47), 55 (22).

*(Z)-13-Hexadecen-11-ynyl Dithioacetate (**5**).* In a three-neck 25-ml round-bottomed flask provided with a gas inlet, septum and magnetic stirrer were placed 3 ml of anh. THF and 0.25 ml (4 mmol) of CS_2. The mixture was cooled to −40°C and a solution of 0.45 ml of 1.4 M MeLi in ether (0.64 mmol) was added under A. The reaction mixture was stirred for 20 min and, after this period of time, a solution of 110 mg (0.32 mmol) of iodo derivative **9** in 2 ml of THF–HMPT (1 : 1) was added and the mixture stirred at room temperature for 4 hr. After quenching with brine and extraction with ether, the combined organic phases were washed with brine and dried ($MgSO_4$) to yield an oily residue, which was chromatographed on silica gel eluting with hexane–ether 6 : 1 to obtain 80 mg (81%) of dithioester **5** (Scheme 3).

IR: ν3020, 2940, 1589, 1200, 710 cm^{-1}. [^{1}H]NMR: δ 5.78 (dt, 1H, J = 10.5 Hz and J = 7 Hz, $CH_2C\underline{H}{=}CH$), 5.38 (d, 1H, J = 10.5 Hz, $CH_2CH{=}C\underline{H}$), 2.5 (t, 2H, J = 7 Hz, CH_2SCS), 2.4 (s, 3H, CH_3), 2.25 (c, 4H, $C\underline{H}_2CH{=}CHC{\equiv}CC\underline{H}_2$), 1.4 (b, 16H, $8CH_2$), 0.95 (t, 3H, J = 7 Hz,



SCHEME 3.

CH_3). [^{13}C]NMR: δ 217 (CS), 144.0 (C-14), 108.8 (C-13), 94.5 (C-11), 78.6 (C-12), 74.4 (C-1), 40.7 (C-1′), 28.4-24.5 (C-2 to C-9 and C-15), 19.5 (C-10), 13.5 (C-16). MS (EI) *m/z* (relative intensity: 310 (M^+, 1), 246 (4), 149 (7), 135 (30), 121 (28), 107 (31), 94 (100), 81 (20), 79 (79), 67 (24), 55 (21).

*1-*tert*-Butyldimethylsilyloxydodec-11-yne (**10**).* This compound was prepared by condensation of lithium acetylide with the corresponding 10-bromo-1-*tert*-butyldimethylsilyloxydecane using a similar method to that described by us (Camps et al., 1983). The required starting material, in turn, was obtained by protection of 10-bromodecan-1-ol with *tert*-butyldimethylsilyl chloride in the presence of triethylamine and *N,N′*-dimethylaminopyridine (Heathcock and Jarvi, 1982).

IR: ν 3400, 2920, 1550, 1250, 1100, 1000, 840 cm^{-1}. [^{1}H]NMR: δ 3.68 (t, 2H, J = 6 Hz, CH_2O), 2.2 (c, 2H, $C\equiv CCH_2$), 1.9 (t, 1H, J = 2.7 Hz, $\underline{H}C\equiv CCH_2$), 1.35 (b, 16H, $8CH_2$), 0.9 (s, 9H, $(CH_3)_3CSi$), 0.05 (s, 6H, $2CH_3Si$).

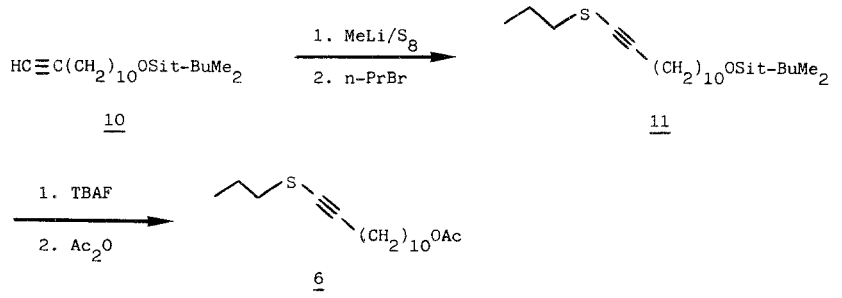
*12-Propylthio-1-*tert*-butyldimethylsilyloxydodec-11-yne (**11**).* In a three-neck round-bottomed flask provided with a nitrogen inlet, septum, and stirring bar, were placed at −70°C 300 mg (1.01 mmol) of protected acetylene **10** and 4 ml of anh. THF. To the solution was added 0.85 ml of a 1.2 M BuLi (1.02 mmol) in hexane and the mixture stirred for 30 min. Then, 34 mg (1.06 mat.g.) of S_8 was added and the mixture stirred at −70°C for 1 hr and at room temperature for 1 hr more. The resulting thiolate was cooled to −40°C, and a solution of 138 mg (1.1 mmol) of *n*-propylbromide in 1 ml of anh. THF was slowly added. The mixture was stirred for 4 hr, quenched with water and thoroughly extracted with ether. The organic phases were washed with brine and dried ($MgSO_4$) to leave, after evaporation of the solvent, a residue that was chromatographed on alumina III eluting with hexane–ether 95:5 to yield 300 mg (82%) of the expected compound **11**.

IR: ν 2920, 2850, 1460, 1260, 1090, 840 cm^{-1}. [^{1}H]NMR: δ 3.6 (t, 2H, J = 6 Hz, CH_2OSi), 2.7 (t, 2H, J = 6.5 Hz, CH_2S), 2.25 (t, 2H, J = 6 Hz, $SC{\equiv}CCH_2$), 1.4 (b, 18H, $9CH_2$), 1.0 (t, 3H, J = 7 Hz, CH_3).

12-(Propylthio)dodec-11-ynyl Acetate (6). To a solution of 300 mg (0.82 mmol) of **11** in 4 ml of anh. THF, cooled in an ice bath, was added 630 mg (2 mmol) of tetrabutylammonium fluoride trihydrate. The mixture was stirred for 30 min at 0°C and 1 hr at room temperature. The solvent was stripped off and the residue chromatographed on alumina III eluting with hexane–ether 5:1 to yield 180 mg (90%) of the corresponding alcohol. Acetylation was carried out in the presence of 1 ml of acetic anhydride and 1 ml of anh. pyridine at room temperature for 5 hr. After quenching with methanol and evaporation of the solvent, the organic material was taken up in ether and washed with brine and dried. The residue was purified by column chromatography on alumina III eluting with hexane–ether 7:2 to afford 180 mg (92%) of acetate **6** (Scheme 4).

IR: ν 2920, 2850, 1735, 1460, 1250, 1220, 1040 cm^{-1}. [^{1}H]NMR: δ 4.05 (t, 2H, J = 6.5 Hz, CH_2OCO), 2.7 (t, 2H, J = 6.5 Hz, CH_2S), 2.3 (t, 2H, J = 6 Hz, $SC{\equiv}CCH_2$), 2.05 (s, 3H, $OCOCH_3$), 1.4 (b, 18H, $9CH_2$), 1.0 (t, 3H, J = 7 Hz, CH_3). MS (EI) *m/z* (relative intensity): 298 (M^+, 1), 189 (11), 173 (13), 149 (12), 143 (18), 135 (15), 119 (13), 105 (18), 94 (100), 79 (46), 66 (14), 55 (19).

13-Tetrahydropyranyloxytridec-2-yn-1-ol (13). In a previously flamed three-neck round-bottomed flask provided with a magnetic bar, A inlet, and septum, was placed at −78°C 1.5 g (5.6 mmol) of 1-tetrahydropyranyloxydodec-11-yne (Camps et al., 1983) in 4 ml of anh. THF. To the solution was slowly added 0.52 ml of a 1 M MeLi (0.52 mmol) in hexane, and the mixture stirred at −78°C for 2 hr. Then, 0.180 g (6 mmol) of paraformaldehyde was slowly added and the mixture stirred for 3 hr at −40°C and 3 hr more at room temperature. After quenching with brine and extraction with ether, the combined organic phases were washed with brine and dried ($MgSO_4$). The solvent



SCHEME 4.

was removed under vacuum to leave the protected alcohol **13** (1.2 g, 80%), pure enough to be used in the next step of the synthesis.

IR: ν 3300, 3280, 2920, 1450, 1210, 1140, 1030, 910 cm^{-1}. [^{1}H]NMR: δ 4.55 (b, 1H, OCHO), 4.20 (t, 2H, J = 2.5 Hz, $HOC\underline{H_2}C{\equiv}C$), 3.6 (c, 4H, $2CH_2O$), 2.15 (c, 3H, $C{\equiv}C\underline{CH_2}$ and OH), 1.55 (b, 22H, $11CH_2$).

2-(13-Bromotridec-11-ynyloxy)tetrahydropyran (14). This compound was prepared according to a procedure already described by us (Camps et al., 1987). Thus, starting from 0.595 g (2.01 mmol) of compound **13,** 490 mg (72%) of bromo derivative **14** was obtained after purification of alumina III.

IR: ν 3290, 2920, 2220, 1460, 1030 cm^{-1}. [^{1}H]NMR: δ 4.55 (b, 1H, OCHO), 3.95 (t, 2H, J = 2.5 Hz, $BrCH_2C{\equiv}C$), 3.5 (c, 4H, 2 CH_2O), 2.2 (c, 2H, $C{\equiv}CCH_2$), 1.45 (b, 22H, $11CH_2$).

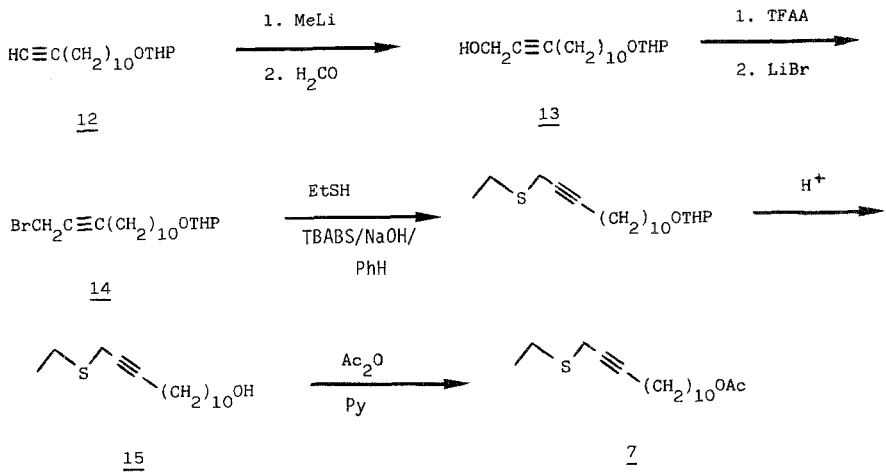
13-(Ethylthio)tridec-11-yn-1-ol (15). A mixture of 450 mg (1.3 mmol) of **14** in 5 ml of anh. benzene, 5 ml of a 20% NaOH solution, 10 mg of tetrabutylammonium bisulfite, and 138 mg (2.22 mmol) of ethanethiol was stirred at room temperature for 4 hr. The phases were decanted and the solvent evaporated off at reduced pressure. The organic material was taken up in 4 ml of methanol and treated with 10 mg of *p*-toluenesulfonic acid for 5 hr. The acid was neutralized with saturated solution of $NaHCO_3$, the solvent stripped off, and the crude product dissolved in ether, washed with brine, and dried. After purification by column chromatography on silica gel eluting with hexane–ether 4:1, 240 mg (79%) of the expected alcohol **15** was obtained.

IR: ν 3300, 2200, 1040 cm^{-1}. [^{1}H]NMR: δ 3.6 (t, 2H, J = 6 Hz, CH_2O), 3.35 (t, 2H, J = 2.5 Hz, $SCH_2C{\equiv}C$), 2.8 (q, 2H, J = 7 Hz, SCH_2CH_3), 2.2 (c, 2H, $C{\equiv}CCH_2$), 1.5 (b, 19H, $8CH_2$ and CH_3).

13-(Ethylthio)tridec-11-ynyl Acetate (7). To a solution of 200 mg (0.78 mmol) of alcohol **15** in 2 ml of anh. pyridine was added 2 ml of acetic anhydride. The mixture was stirred at room temperature for 5 hr, cooled on an ice bath, and quenched with 2 ml of methanol. After stirring for 15 min, the volatile material was evaporated under vacuum and the residue taken up in ether, washed with brine, and dried. The product was purified by column chromatography on silica gel eluting with hexane–ether 4:1 to yield 210 mg (90%) of acetate **7** (Scheme 5).

IR: ν 3300, 2920, 2200, 1740, 1450, 1230, 1040 cm^{-1}. [^{1}H]NMR: δ 4.05 (t, 2H, J = 6.5 Hz, CH_2OCO), 3.3 (t, 2H, J = 2.5 Hz, $SCH_2C{\equiv}C$), 2.8 (q, 2H, J = 7 Hz, $CH_3C\underline{H_2}S$), 2.25 (c, 2H, $C{\equiv}CCH_2$), 2.05 (s, 3H, $OCOCH_3$), 1.45 (b, 19H, $8CH_2$ and CH_3). MS (EI) m/z (relative intensity): 298 (M^+, <1), 269 (2), 209 (3), 153 (1), 141 (2), 139 (3), 125 (5), 114 (67), 97 (12), 85 (100), 79 (13), 67 (10), 55 (13).

1-Bromo-(Z)-12-pentadecen-10-yne (17). This compound was prepared according to the method already reported by us (Camps et al., 1987). Thus, starting from 300 mg (1.35 mmol) of alcohol **16,** prepared by sequential pal-



SCHEME 5.

ladium-catalyzed coupling reaction of (Z)-1-bromo-1-butene and 2-(10-undecynyloxy)tetrahydropyran (Michelot et al., 1982) followed by acid hydrolysis, 330 mg (89%) of bromide **17** was obtained after purification on silica gel eluting with hexane.

Anal.: Calcd. for $C_{15}H_{25}Br$: C, 63.16; H, 8.77. Found: C, 63.05; H, 8.81. IR: ν 3020, 2980, 1610, 1450, 720 cm^{-1}. [^{1}H]NMR: δ 5.80 (dt, 1H, J = 10.5 Hz and J = 7 Hz, $CH_2C\underline{H}{=}CH$), 5.4 (d, 1H, J = 10.5 Hz, $CH_2CH{=}C\underline{H}$), 3.45 (t, 2H, J = 6 Hz, CH_2Br), 2.2 (c, 4H, $CH_2C{=}C$ and $CH_2C{\equiv}C$), 1.4 (b, 14H, $7CH_2$), 1.0 (t, 3H, J = 7 Hz, CH_3). MS (EI) *m/z* (relative intensity): 286 (M^++2, 38), 284 (M^+, 40), 257 (20), 175 (14), 149 (20), 135 (55), 121 (20), 107 (60), 93 (69), 79 (100), 67 (23), 55 (15).

*(Z)-12-Pentadecen-10-ynethiol (**19**).* To a solution of 179 mg (0.268 mmol) of bromide **17** in 2 ml of ethanol was added 50 mg (0.27 mmol) of thiourea. The mixture was heated to reflux for 2 hr, cooled, and the resulting salt **18** subjected to hydrolysis by reaction with 43 mg (1.1 mmol) of NaOH in 1 ml of water at room temperature for 24 hr. After extraction with benzene, the combined organic phases were washed with brine and dried. The solvent was evaporated off and the residue purified on silica gel to afford 120 mg (78%) of thiol **19.**

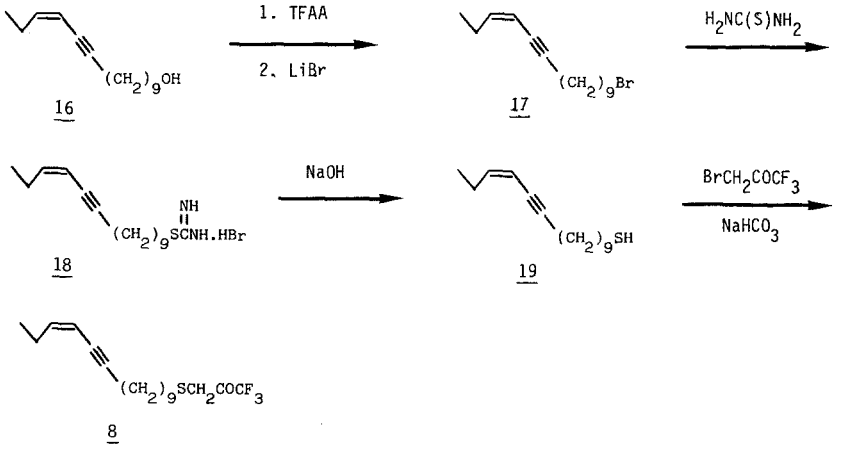
IR: ν 3020, 2980, 2590, 1450, 720 cm^{-1}. [^{1}H]NMR: δ 5.80 (dt, 1H, J = 10.5 Hz and J = 7 Hz, $CH_2C\underline{H}{=}CH$), 5.40 (d, 1H, J = 10.5 Hz, $CH_2CH{=}C\underline{H}$), 2.45 (t, 2H, J = 6.8 Hz, CH_2S), 2.15 (c, 4H, $C\underline{H}_2CH{=}CH{-}C{\equiv}C\underline{CH}_2$), 1.4 (b, 14H, $7CH_2$), 1.0 (t, 3H, J = 7 Hz, CH_3). MS (EI) *m/z* (relative intensity): 238 (M^+, 7), 209 (15), 195 (10), 167 (12),

135 (18), 121 (13), 106 (22), 94 (85), 80 (22), 79 (100), 67 (40), 61 (12), 55 (29).

3-[(Z)-12-Pentadecen-10-ynylthio]-1,1,1-trifluoropropan-2-one (8). To a solution of 120 mg (0.5 mmol) of thiol **19** in 4 ml of anh. Cl_2CH_2 were added 200 mg (1.88 mmol) of sodium bicarbonate and 144 mg (0.75 mmol) of 3-bromo-1,1,1-trifluoropropan-2-one. The mixture was stirred at room temperature for four days, filtered, and the solvent removed under vacuum. The residue was purified on column chromatography over silica gel to afford 110 mg (65%) of the expected ketone **8** (Scheme 6).

IR: ν 3020, 2980, 1745, 1460, 1175, 1130, 720 cm^{-1}. [^{1}H]NMR: δ 5.8 (dt, 1H, J = 10.5 Hz and J = 7 Hz, $CH_2C\underline{H}{=}CH$), 5.45 (d, 1H, J = 10.5 Hz, $CH_2CH{=}C\underline{H}$), 3.48 (s, 2H, SCH_2CO), 2.35 (t, 2H, J = 7 Hz, $C\underline{H}_2SCH_2CO$), 2.1 (c, 4H, $C\underline{H}_2CH{=}CHC{\equiv}CC\underline{H}_2$), 1.4 (b, 14H, $7CH_2$), 0.95 (t, 3H, J = 7 Hz, CH_3). MS (EI) *m/z* (relative intensity): 348 (M^+, 12), 279 (8), 183 (3), 169 (8), 156 (4), 149 (4), 140 (3), 135 (8), 121 (9), 107 (17), 94 (100), 79 (52), 67 (20), 55 (13). [^{19}F]NMR: δ −76.4 (s).

Laboratory Bioassays. Inhibition experiments were carried out by placing 1- to 2-day old males in 10-cm-ID Petri dishes. The dishes contained a 2 × 2-cm piece of Whatman No. 1 filter paper on which several amounts of the test compounds (0.1, 1, 10, 100, and 1000 μg) in hexane had been applied. The solvent was evaporated off immediately before the assay. A similar piece of paper treated with 100 μl of hexane and evaporated to dryness was used as control. The males were subjected to the vapors of the compounds for 4 hr in the dark, then taken out and after 5 min their antennae removed. Electroantennogram activity was determined on a EAG set up as previously described (Guer-



SCHEME 6.

rero et al., 1986). Ten "puffs" with 10 μg of synthetic pheromone were directed over the excised antennae and the depolarizations recorded at 40-s intervals to ensure full recovery of the antennal receptors. The EAG values were normalized to obviate the time-dependent changes in antennal responses. Between three and six replicates were carried out for each test. Inhibition values were obtained from the expression:

$$\% \text{ inhibit.} = \frac{\text{mean EAG pheromone response} - \text{mean EAG ph. response after presaturation}}{\text{mean EAG pheromone response}} \times 100$$

and the results were analyzed statistically for significance according to the Student's t test.

Field Tests. The required amount of test compound in each bait was mixed with 2.5 mg of paraffin, in order to slow down the release rate in the field, and dissolved in 1 ml of nanograde hexane. The solutions were transferred into closed polyethylene vials (3 × 1.1 cm ID), which were used as dispensers. Field trials were conducted in Mora de Rubielos (Teruel, Spain) during the 1987–1988 seasons. Traps used for the experiments were "dry" and specially designed for processionary moth catches (Montoya, 1984). Traps were hung on trees at a height of 1.7–2.0 m and spaced 50 m apart when belonging to the same parcel. They were set out in statistically randomized blocks and revised and rotated every day. Five traps were used for each formulation. Trap catches were subjected to a square-root transformation followed by analysis of variance, and the data were analyzed statistically for significance according to Duncan's multiple-range test.

RESULTS AND DISCUSSION

As we have mentioned above, we have prepared new sulfur analogs of the pheromone structure **1** and studied their biological activity in laboratory and field assays. Synthesis of thioester **3** was accomplished by the esterification reaction of (*Z*)-13-hexadecen-11-yn-1-ol (**2**) with thioacetic S-acid in the presence of triphenylphosphine-diisopropyl azodicarboxylate (Volante, 1981) in 89% yield. Compound **4** was synthesized in a one-step process by preparation of the trimethylsilyl enol ether of *pityolure* **1** followed by reaction with dry H_2S in 69% overall yield from **1** (Corey and Wrigth, 1984). It must be noted that reaction of **1** with Lawesson reagent, 2,4-bis(*p*-methoxyphenyl)-1,3-dithiadiphosphetane-2,4-disulfide, considered one of the most efficient thionation reagents of ketones, amides, and esters (Pedersen et al., 1978), did not give in our hands acceptable yields of the expected thioester **4** (Scheme 2).

Dithioester **5** was obtained by alkylation of iodide **9** with lithium dithioacetate in 81% yield, prepared in situ by the action of methyllithium on carbon

disulfide at low temperature (Meijer et al., 1973). Again, direct thionation reaction of thioester **3** with Lawesson reagent to obtain compound **5** did not yield the expected product (Scheme 3). On the other hand, syntheses of compounds **6** and **7** were successfully accomplished starting from the same substrate, dodec-11-yn-1-ol, which was protected as the *tert*-butyldimethylsilyl ether **10** or the tetrahydropyranyl ether **12.** Reaction of the acetylide of **10** with S_8 afforded the corresponding thiol, which was alkylated in situ to the corresponding sulfide **11.** After hydrolysis and acetylation, the required compound **6** was obtained in 75% overall yield from **10** (Scheme 4). Similarly, reaction of the acetylide of **12** with paraformaldehyde furnished alcohol **13,** which was transformed into bromide **14** (Camps et al., 1987). Reaction of **14** with ethanethiol under phase-transfer catalytic conditions yielded, after hydrolysis, alcohol **15,** which was finally acetylated to afford acetate **7** in 41% overall yield from **12** (Scheme 5). Trifluoromethylketone **8** was prepared, according to the procedure outlined in Scheme 6, by reaction of bromide **17** with thiourea to yield, after hydrolysis, thiol **19,** which was coupled with 3-bromo-1,1,1-trifluoropropan-2-one in anhydrous THF in the presence of sodium bicarbonate. The overall yield of the process was 45% from **16.**

In laboratory bioassays, inhibition was tested by recording the EAG response of the natural pheromone when the insects were treated with vapors of variable amounts of the synthetic pheromone and analogs. As shown in Figures 1–3, the inhibition of the EAG responses was plotted versus amount of compounds tested. The most effective doses were in the 100 to 1000-μg range, whereas the lower concentrations (0.1 and 1 μg) were in most cases inefficient. As expected, the highest inhibition effect was shown by the synthetic pheromone **1,** which even at 0.1 μg displayed a high level of activity. On the other hand, higher doses promoted a U-shaped curve with an inflection point of minimum activity (55%) at 10 μg. Among the pheromone analogs assayed, compounds **3** and **6** were the most effective (70–83%), with a similar inhibition level shown by thioester **3** in comparison with the pheromone **1** at the 10- and 100-μg doses (Figure 1). Compounds **4, 5,** and **8** showed only moderate activity at 1000 μg (18–35%), whereas compound **7** was practically inactive. On the other hand, in agreement with the results of field trials (see below), the intrinsic EAG responses of the different analogs are relatively low compared with that of the pheromone (Figure 4).

It is noteworthy that, whereas replacement of the oxygen atom of the alkoxy group by sulfur in the ester moiety of the pheromone (compound **3**) leads to the highest inhibitory activity, only a very modest effect appears when the corresponding oxygen atom of the carbonyl group is exchanged (compound **4**). If both modifications are carried out simultaneously, the resulting compound **5** shows an intermediate inhibitory action.

When trifluoromethylketone **8** was assayed as a pheromone perception

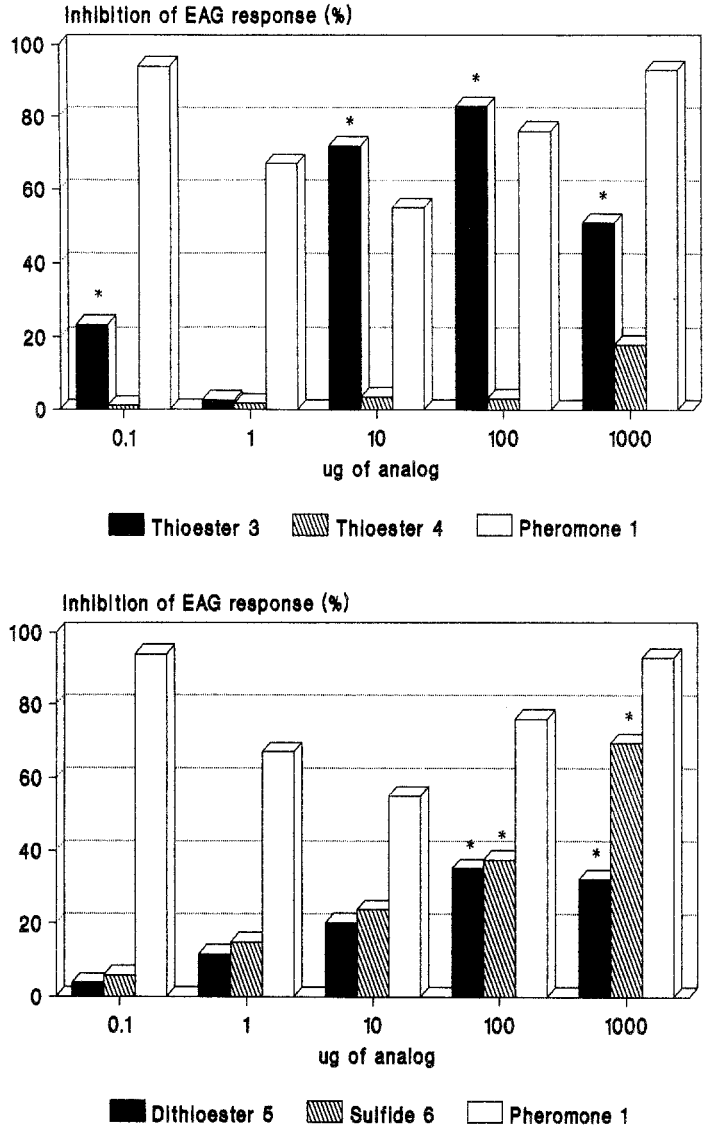


FIG. 1–3. Inhibition of the natural pheromone EAG response promoted by analogs **3–8.** Bars represent the mean relative response (%) of six experiments. Responses with an asterisk (*) are statistically significant at $P < 0.05$ (Student's t test).

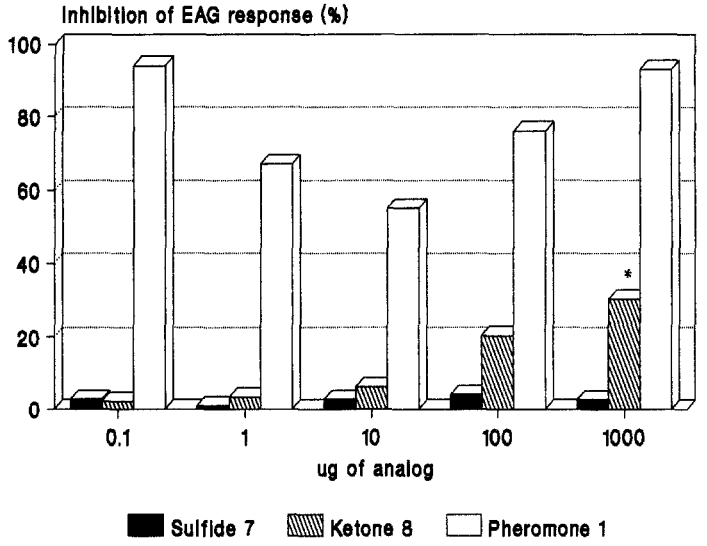


FIG. 1–3. Continued.

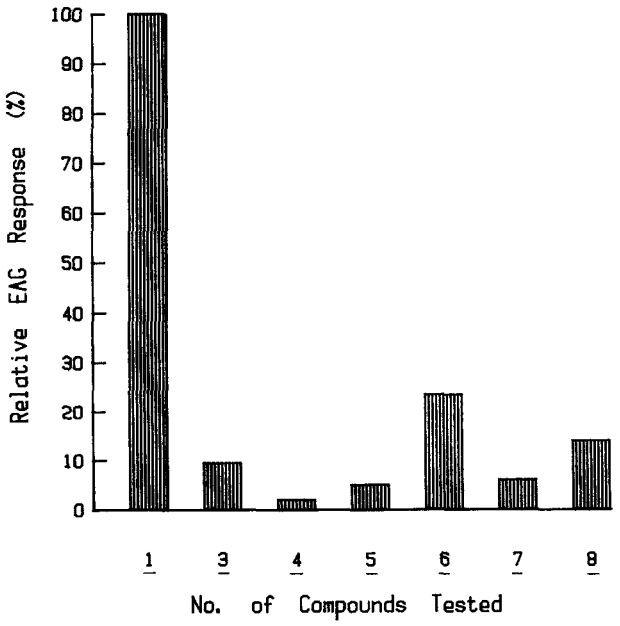


FIG. 4. Relative intrinsic EAG activity showed by 10 μg of compounds **3–8.**

inhibitor in the laboratory bioassay, only moderate activity was found (30% at 100- to 1000-μg concentrations). However, in the field, a marked enhancement of this effect (95% inhibition) was observed when this compound was mixed with the pheromone in a 10:1 ratio (Table 1). This preliminary result may encourage the pursuit of more extensive work on this possible new application of trifluoromethylketones in pest control studies.

Likewise, in field assays, thioester **4** and dithioester **5** showed a very high inhibition activity, ranging from 85 to 97%, when mixed with the pheromone is 0.1:1, 1:1, and 10:1 ratios (Table 2), whereas, perhaps surprisingly, compound **3** was only moderately active. Sulfides **6** and **7** behaved as good inhibitors also when applied in baits containing a mixture of inhibitor–pheromone in 1:1 and 10:1 ratios. In agreement with the above EAG results, these compounds showed a very low attractant activity when used alone.

In summary, sulfur analogs **3–8,** which formally proceed from structural modifications on two of the three putative critical molecular active sites of *pityolure* **1,** have been prepared for the first time and appeared to be good antagonists of the pheromone action in laboratory and field assays. In the laboratory, compounds **3** and **6** appeared to cause a marked decrease in the EAG response

TABLE 1. INHIBITORY EFFECTS OF COMPOUNDS **6–8** ON PITYOLURE **1** (MORA DE RUBIELOS, 1988)

	Bait formulation (mg)					
Parcel[a]	Sulfide **6**	Sulfide **7**	Ketone **8**	Pityolure	Total No. of catches[b]	Relative inhibitory activity (%)
IV	0.1			1	280 b	15
	1			1	137 c	58
	10			1	126 c	62
				1	330 a	
	1				53 c	(16)[c]
V		0.1		1	379 b	16
		1		1	303 c	33
		10		1	211 c	53
				1	453 a	
		1			3 d	(0.7)[c]
VI			0.1	1	234 a	29
			1	1	86 b	74
			10	1	15 c	95
				1	329 a	
			1		2 d	(0.6)[c]

[a] Several km of distance between parcels.
[b] Five replicates per trap. Catches followed by the same letter are not significantly different at $P = 0.05$ (Duncan's multiple-range test).
[c] Relative intrinsic attractant activity (%) of the analog compared with pityolure.

TABLE 2. INHIBITORY EFFECTS OF COMPOUNDS 3–5 ON PITYOLURE 1 (MORA DE RUBIELOS, 1987–1988)

Parcel[a]	Bait formulation (mg)				Total No. of catches[b]	Relative inhibitory activity (%)
	Thioester 3	Thioester 4	Dithioester 5	Pityolure		
I	0.1			1	309 a	−10[c]
	1			1	214 bc	24
	10			1	169 bcd	40
				1	281 ab	
	1				0 e	(0)[d]
II		0.1		1	56 b	85
		1		1	24 b	93
		10		1	16 b	96
				1	382 a	
		1			12 b	(3)[d]
III			0.1	1	43 b	88
			1	1	10 b	97
			10	1	16 b	95
				1	365 a	
			1		0 c	(0)[d]

[a] Several km of distance between parcels.
[b] Five replicates per trap. Catches followed by the same letter are not significantly different at $P = 0.05$ (Duncan's multiple-range test).
[c] An enhancement of the trap catch was obtained in this case.
[d] Relative intrinsic attractant activity (%) of the analog compared with pityolure.

of the male processionary moth *Thaumetopoea pityocampa* to the natural pheromone. Compounds **4, 5,** and **8** were, in turn, moderately active in the laboratory, whereas in the field they behaved as very good antagonists of the pheromone action amounting the inhibition level to 97%.

According to our results, replacement of either one of the oxygen atoms of the acetate group in *pityolure* by sulfur results, in both cases, in a marked inhibitory action of the resulting analog. On the other hand, replacement of a CH group of the ethylenic moiety by S in α position to the triple bond (compound **6**) causes a much higher inhibition effect at all doses than when substitution takes place in β position (compound **7**). This finding and the relatively higher intrinsic EAG activity of **6** in relation to **7** seems to suggest that the presence of electron-donating atoms like S in α to the triple bond might lead to compounds with interesting inhibitory pheromonal activity. Further work on this line is in progress in our laboratory.

Although at present we have no evidence of the physiological mode of action of the analogs tested, several mechanisms can be hypothesized. In the

laboratory tests, the EAG results can be explained by a long-lasting adaptation of electrical responses of the receptor cells or by habituation of the central nervous system to the incoming signals. In the field, on the other hand, the inhibitory results might be due to an improper activation of other receptor cell types by the corresponding analog. In any case, the possibility of an irreversible binding of the inhibitors to the receptor proteins cannot be ruled out.

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CUTICULAR HYDROCARBONS OF EIGHT SPECIES OF NORTH AMERICAN CONE BEETLES, *Conophthorus* HOPKINS[1]

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Abstract—A study to determine the degree of similarity and/or diversity among eight of the 15 described species of *Conophthorus* is reported. Cuticular hydrocarbons were evaluated for *C. conicolens*, *C. ponderosae*, *C. cembroides*, *C. edulis*, *C. radiatae*, *C. coniperda*, *C. resinosae*, and *C. banksianae*. Seventy-eight individual and isomeric mixtures of hydrocarbons were identified by gas chromatography–mass spectrometry, including *n*-alkanes, alkenes, alkadienes, 2- or 4-methylalkanes, 3-methylalkanes, and single-component and isomeric mixtures of internally branched mono-, di-, and trimethylalkanes. Differences in alkenes and mono-, di-, and trimethylalkanes can be used easily to separate the eight species. *Conophthorus conicolens* and *C. ponderosae* contain the most complex blends. Hydrocarbon patterns in three geographically separated populations of *C. ponderosae*, each from a different host, are qualitatively identical with the exception of a homologous series of 3,7-dimethylalkanes from adults collected from *Pinus lambertiana* cones. The latter could comprise a sibling species. Hydrocarbon mixtures of two eastern species, *C. resinosae* and *C. banksianae*, are qualitatively identical, supporting the suspicion that *C. banksianae* may not be a valid species. Closely related *C. cembroides* and *C. edulis* have similar combinations of hydrocarbons except for a unique and abundant alkene ($C_{27:1}$) in *C. edulis* and two dimethyhexacosanes in *C. cembriodes*.

[1] Coleoptera: Scolytidae.

Key Words—Cuticular lipids, chemotaxonomy, methyl-branched hydrocarbons, mass spectra, *Pinus* species, Coleoptera, *Conophthorus*, Scolytidae, insect integument.

INTRODUCTION

Beetles of the genus *Conophthorus* Hopkins (family Scolytidae) attack and kill female cones of *Pinus* species during the second year of cone development. In severe infestations up to three-fourths of the cones can be destroyed (Furniss and Carolin, 1977). Such sizable reductions in seed crops adversely affect natural and managed reforestation. Most species of the genus are monophagous; the most dramatic exception is *Conophthorus ponderosae* Hopkins. This polyphagous species reportedly breeds in cones of 8–13 species of *Pinus* and ranges from northern British Columbia to southern Mexico (Hedlin et al., 1980; Wood, 1982).

Wood (1982) synonomized *C. ponderosae* with five other species of *Conophthorus*: *C. scopulorum* Hopkins, *C. contortae* Hopkins, *C. monticolae* Hopkins, *C. flexilis* Hopkins, and *C. lambertianae* Hopkins. Previous species diagnoses were generally made on the basis of host, geography, and morphological features. "Taxonomic characters in *Conophthorus* species are so few and poorly developed that a search for non-conventional approaches is needed in order to find a means for the accurate identification of species" (S. L. Wood, personal communication). Wood (1982) found the collected material difficult to interpret. Perhaps *C. ponderosae* is in the process of speciation or it comprises two or more sibling species awaiting discovery. Wood (1982) candidly admits ". . . material from five-needle pines could be different."

Future control methods for *Conophthorus* will likely rely on mate-finding and host-acceptance behaviors, which are presumably under genetic control and are assumed to be species specific. Thus proper identification and understanding of the biology of *Conophthorus* species are fundamental to development of sound pest management programs. Since many of the *Conophthorus* species are difficult to separate morphologically, we decided to evaluate cuticular hydrocarbons as another set of taxonomic characters.

In this paper we report the identification of the hydrocarbon components in the cuticular wax of eight species of *Conophthorus*. This is the first step in determining the degree of similarity and/or diversity of cuticular hydrocarbon patterns among the species of this genus.

METHODS AND MATERIALS

Adult cone beetles characterized in this paper were collected as overwintering adults from infested cones or twig terminals. *C. ponderosae* was collected from three different species of pine: sugar pine, *Pinus lambertiana*

Dougl.; ponderosa pine, *P. ponderosae* Dougl. ex Laws.; and western white pine, *P. monticola* Dougl. All other *Conophthorus* were collected from a single host: *C. conicolens* Wood from Chihuahua pine, *P. leoiphylla* var. *chihuahua* (Engelm.) Shaw; *C. cembroides* Wood from Mexican pinyon pine, *P. cembroides* Zucc.; *C. edulis* Hopkins from pinyon pine, *P. edulis* Engelm.; *C. coniperda* (Schwartz) from eastern white pine, *P. strobus* L.; *C. radiatae* Hopkins from Monterey pine, *P. radiata* D. Don; *C. banksianae* McPherson from Jack pine, *P. banksiana* Lamb.; and *C. resinosae* Hopkins from red pine, *P. resinosa* Ait.

Pine cones infested with *Conophthorus* are not always easy to collect. Cones of sugar pine, western white pine, and eastern white pine fall to the ground at the end of the summer. Overwintering, adult *Conophthorus* remain in the cones on the ground and emerge as parent adults in the spring. Infested cones of these species are relatively easy to collect; cones are simply picked up off the ground and placed in a collection bag. Cones of Monterey pine, ponderosa pine, and Chihuahua pine are difficult to collect. Even after they are infested, they are retained in the crown of the tree for one or more seasons. By the time trees present their infested cones to the biologist (i.e., drop them on the ground), the beetles have already emerged. Cones are usually borne in the upper portions of the crown of trees ≥ 20 m in height. Biologists must resort to the use of a pole pruner to cut the cones down, a rifle to shoot the cones down, or we must collect from sexually mature, small trees or downed, mature trees in logging operations. Cones of the pinyon pines are relatively easy to collect; the trees are short (≤ 6 m in height) and bushy, and trees with cones usually have them distributed throughout the crown.

Hydrocarbon mixtures of *Conophthorus* reported in this paper represent data obtained from adult beetles that emerged from cones collected from the following locations: sugar pine from the Eldorado National Forest near Placerville, California; ponderosa pine from the Sierra National Forest ca. 40 km south of Yosemite National Park, California; western white pine from Sandpoint, Idaho; Chihuahua pine and Mexican pinyon pine cones from the Chiricahua Mountains in southern Arizona; pinyon pine from Prescott, Arizona and the Chiricahua Mountains; eastern white pine from Murphy, North Carolina and Petawawa, Ontario, Canada; Monterey pine from Albany and Aptos, California; jack pine from Aubrey Falls, Ontario, Canada, and red pine from Little Rapids, Ontario, Canada.

Infested cones from the western pine species were collected by three of the authors; cones from the eastern species were supplied by colleagues. In addition, we have examined cuticular hydrocarbons from museum specimens. Some of these beetles were collected before 1920; hydrocarbon patterns of these specimens are, remarkably, qualitatively identical to those collected and extracted by the authors. Hydrocarbon patterns of many of the *Conophthorus* species presented in this paper are representative of additional beetle collections made by the authors and of museum specimens.

Beetles were allowed to emerge from the cones or twigs. One to three days after emergence beetles were frozen and held at −20°C until their hydrocarbons were extracted. Beetles were removed from the freezer and allowed to warm to ambient temperature. Cuticular lipids were then extracted by immersing 15–50 beetles as a group once in 10 ml of hexane for 10 min. Sexes were not separated; males and females were extracted together. Extracted beetles were prepared as voucher specimens and submitted to the U.S. National Museum, Smithsonian Institution, Washington, D.C. After beetles were extracted, hydrocarbons were separated from other components by pipetting the 10-ml extract and an additional 8 ml of hexane through 3 cm of activated BioSil-A in Pasteur pipet minicolumns (Blailock et al., 1976). All hydrocarbon extracts were evaporated to dryness under a stream of nitrogen and redissolved in 30 μl of hexane for analysis.

Gas chromatography–mass spectrometry (GC-MS) analyses were performed on Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 5970B Mass Selective Detector, which was interfaced with a Hewlett Packard Chemstation computer. The GC-MS system was equipped with a fused silica capillary column (30 m × 0.2 mm ID, HP-1) and operated in split mode (with a split ratio of 20:1). Each mixture was analyzed by a temperature program from 200°C to 320° at 3°C/min with a final hold of 20 min. Electron impact (EI) mass spectra were obtained at 70 eV. Retention times and mass spectra of extracted *n*-alkanes were compared with external standards. Alkenes and methyl-branched alkanes were tentatively identified by calculating their equivalent chain lengths (ECL) (Nelson and Sukkestad, 1970; Jackson and Blomquist, 1976). Mass spectra of alkenes and methlyalkanes were interpreted as described by Nelson et al. (1972), Nelson (1978), Pomonis et al. (1978), and Blomquist et al. (1987).

RESULTS

Data presented in this report are the results of pooled samples (mixed sexes), from numerous cones, from several trees of the same species, from any location. In preliminary studies with other scolytid beetles, *Dendroctonus brevicomis* LeConte and *D. frontalis* Zimmerman, we determined that the hydrocarbon blends of pupae and callow adults are incomplete when compared to adults. However, hydrocarbon mixtures of fully tanned, emerged adults are fixed and do not change over the short lifetime of the beetles. In early, unpublished studies of *C. ponderosae*, we determined that there were no qualitative differences within beetles collected from the same host from several disparate geographical locations. Furthermore, we examined groups of males and females of *C. banksianae* and *C. resinosae* and found no qualitative differences in their hydrocarbon profiles. Therefore, we feel justified in presenting results of pooled samples of each species of *Conophthorus*.

All of the major hydrocarbon components (mean percent $\geq 0.5\%$ of the total hydrocarbon mixture) in the cuticular wax of eight species of *Conophthorus* were characterized (Table 1). Hydrocarbon components consist of homologous series of *n*-alkanes, alkenes, alkadienes, 2- or 4-methylalkanes, 3-methylalkanes, and single-component and isomeric mixtures of internally branched monomethylalkanes, dimethylalkanes, and trimethylalkanes. Unique and abundant species-specific hydrocarbon components were identified. Monomethyl-, dimethyl-, trimethylalkanes and unsaturated components can easily be used to separate the eight species of *Conophthorus* (Table 2). GC-MS total ion chromatograms for each species are displayed; closely related species, as inferred from Wood's (1982) key, are paired on the same figure (Figures 1–4).

The *n*-alkane composition in all species, except *C. conicolens*, is a continuous series from C_{21} (*n*-heneicosane) to C_{31} with C_{23}, C_{25}, and C_{27} predominating. In *C. conicolens* the *n*-alkane series includes small quantities of C_{32}, C_{33}, C_{35}, and C_{37}. All species have measurable quantities of *n*-alkanes of even-numbered chain length from C_{22} to C_{28}; C_{24} and C_{26} are the most abundant.

Alkenes were identified by their retention times and diagnostic mass ions. The molecular ion of an alkene is 2 mass units less than the molecular ion of the corresponding *n*-alkane. Alkadienes produce a molecular ion which is 4 mass units less. No structural information, position of double bond(s), or stereochemistry was obtained on alkenes. Each *Conophthorus* species contains $C_{25:1}$ (pentacosene) and $C_{27:1}$. *C. ponderosae* populations possess alkenes from $C_{23:1}$ to $C_{35:1}$. The major alkene in *C. conicolens*, *C. ponderosae*, *C. cembroides*, C. radiatae, *C. resinosae*, and *C. banksianae* is $C_{25:1}$, whereas $C_{27:1}$ is most abundant in *C. edulis* and *C. coniperda*. *C. resinosae* and *C. banksianae* both contain significant amounts of $C_{35:1}$ and $C_{37:1}$. Other alkenes are present in very small or trace amounts and occur only as a shoulder of the peak of a 3-(Me) C_n (3-methylalkane). The hydrocarbons from *C. ponderosae*, *C. coniperda*, *C. resinosae*, and *C. banksianae* contain the only alkadienes, $C_{25:2}$ (pentacosadiene, peak 12) and $C_{27:2}$ (peak 23). The mass spectra of peaks 12 and 23 showed the characteristic molecular ions, *m/z* 348 and 376, respectively, which are 4 mass units less than the molecular ions of C_{25} and C_{27}, respectively.

All *Conophthorus* species, except *C. conicolens*, *C. cembroides*, and *C. edulis*, contain mixtures of hydrocarbons with both 2-(Me) C_n and 3-(Me) C_n. It is difficult to distinguish 2-(Me) from 4-(Me) C_n (Blomquist et al., 1987). Peak 23 (Table 1) from *C. coniperda* illustrates this situation and is interpreted as 2- or 4-(Me) C_{26}. The high-abundance M-43 ion at *m/z* 337 is consistent with 2-(Me) C_{26}. The M-71 : M-72 ion pair from this spectrum, and others, varied in intensity, indicating the possible presence of 4-(Me) C_{26}. Therefore, all are designated as either 2- or 4-(Me) C_n (Tables 1 and 2). The 3-(Me) C_n were identified by their retention times and spectra, which gave a strong $(M-29)^+$ ion and a weaker $(M-57)^+$ ion.

TABLE 1. HYDROCARBONS IDENTIFIED FOR EIGHT *Conophthorus* SPECIES

Peak[a]	Hydrocarbon	ECL[b]	CN[b]	Diagnostic MS ions
1	2-Methyleicosane	20.70	21	252/253, 280/281, 296
2	*n*-Heneicosane	21.00	21	296
3	*n*-Docosane	22.00	22	310
4	2- or 4-; 3-Methyldocosane[c]	22.76	23	252/253, 280/281, 308/309, 324; 295/296, 266/267, 324
5	Tricosene	22.76	23	322
6	*n*-Tricosane	23.00	23	324
7	9-; 11-; 13-Methyltricosane[c]	23.39	24	140/141, 224/225; 168/169, 196/197; *196/197, 168/169*
8	Tetracosene	23.70	24:1	336
9	3-Methyltricosane	23.73	24	280/281, 308/309, 338
10	*n*-Tetracosane	24.00	24	338
11	2- or 4-Methyltetracosane	24.63	25	308/309, 336/337, 352
12	Pentacosadiene	24.69	25:2	348
13	Pentacosene	24.70	25:1	350
14	*n*-Pentacosane	25.00	25	352
15	9-; 11-; 13-Methylpentacosane[c]	25.38	26	140/141, 252/253, 366; 168/169, 224/225, 366; 196/197, 366
16	5-Methylpentacosane	25.50	26	84/85, 308/309
17	11,15-Dimethylpentacosane	25.60	27	168/169, 239
18	2- or 4-Methylpentacosane	25.70	26	323, 351, 366
19	Hexacosene + 3-methylpentacosane[c]	25.73	26:1	364
		25.73	26	308/309, 336/337, 366
20	*n*-Hexacosane	26.00	26	366
21	12-; 13-Methylhexacosane[c]	26.34	27	182/183, 224/225; 196/197, 210/211
22	11,15-; 13,17-Dimethylhexacosane[c]	26.60	28	168/169, 182/183, 239, 253; 196/197, 154/155, 225, 267

23	2- or 4-Methylhexacosane + heptacosadiene[c]	26.63	27 27:2	336/337, 364/365, 308/309, 380 376
24	Heptacosene	26.70	27:1	378
25	3-Methylhexacosane	26.79	27	322/323, 350/351, 380
26	*n*-Heptacosane	27.00	27	380
27	9-; 11-; 13-Methylheptacosane[c]	27.32	28	140/141, 280/281; 168/169, 252/253; 196/197, 224/225, 394
28	9-Methylheptacosane	27.40	28	140/141, 280/281, 394
29	7-Methylheptacosane	27.42	28	112/113, 308/309
30	5-Methylheptacosane	27.50	28	84/85, 336/337, 394
31	9,13-; 9,15-; 11,15-Dimethylheptacosane[c]	27.63	29	140/141, 224/225, 211, 295; 140/141, 196/197, 239, 295; 168/169, 196/197, 239, 267
32	3-Methylheptacosane	27.74	28	336/337, 364/365, 394
33	*n*-Octocosane	28.00	28	394
34	9-; 11-; 13-; 15-Methyloctacosane[c]	28.32	29	140/141, 294/295 168/169, 266/267 196/197, 238/239 210/211, 224/225
35	11,15-; 13,17-Dimethyloctacosane[c]	28.62	29	168/169, 210/211, 239, 281; 196/197, 182/183, 253, 267
36	3-Methyloctacosane	28.70	29	350/351, 378/379, 408
37	*n*-Nonacosane	29.00	29	408
38	9-; 11-; 13-; 15-Methylnonacosane[c]	29.32	30	140/141, 308/309; 168/169, 280/281; 196/197, 252/253; 224/225
39	5-Methylnonacosane	29.50	30	84/85, 364/365
40	11,15-; 13,17-Dimethylnonacosane[c]	29.61	31	168/169, 224/225, 239, 295; 196/197, 267
41	3-Methylnonacosane	29.73	30	364/365, 392/393, 422
42	13,17,21-Trimethylnonacosane	29.90	32	196/197, 140/141, 211, 267, 281, 337
43	*n*-Triacontane	30.00	30	422
44	3,7-Dimethylnonacosane	30.10	31	126/127, 336/337, 407

TABLE 1. Continued

Peak[a]	Hydrocarbon	ECL[b]	CN[b]	Diagnostic MS ions
45	11,15-; 13,17-Dimethyltriacontane[c]	30.60	32	168/169, 238/239, 239, 309; 196/197, 210/211, 267, 281
46	*n*-Hentriacontane	31.00	31	436
47	7-; 9-; 11-Methylhentriacontane[c]	31.40	32	112/113, 364/365; 140/141, 336/337; 168/169, 308/309
48	11,15-; 13,17-Dimethylhentriacontane[c]	31.64	33	168/169, 252/253, 239, 323; 196/197, 224/225, 267, 295
49	Docotriacontene + 3-methyltriacontane[c]	31.71	32:1 32	448 421/422, 392/393, 450
50	11,15,19-Trimethylhentriacontane	31.90	34	168/169, 196/197, 267, 239, 309, 337
51	Docotriacontane	32.00	32	450
52	3,7-Dimethylhentriacontane	32.14	33	126/127, 364/365, 435
53	Tritriacontene	32.75	33:1	462
54	*n*-Tritriacontane	33.00	33	464
55	9-; 11-; 13-; 15-Methyltritriacontane[c]	33.34	34	140/141, 364/365; 168/169, 336/337; 196/197, 308/309; 224/225, 252/253
56	11,19-; 11,15-; 11,21-; 13,17-; 13,19-Dimethyltritriacontane[c]	33.57	35	168/169, 224/225, 295, 351; 168/169, 280/281, 239, 351; 168/169, 196/197, 323, 351; 196/197, 252/253, 267, 323; 196/197, 224/225, 295, 323
57	11,15,21-; 13,17,21-; 13,17,23-Trimethyltritriacontane[c]	33.86	36	168/169, 196/197, 239, 295, 337, 365; 196/197, 267, 337; 196/197, 168/169, 267, 337, 365

58	3,7-Dimethyltritriacontane	34.08	35	126/127, 392/393, 463
59	11-; 12-Methyltetracontane[c]	34.30	35	168/169, 350/351; 182/183, 336/337
60	Pentatriacontene	34.70	35:1	490
61	Pentatriacontane	35.00	35	492
62	9-; 11-; 13-Methylpentatriacontane[c]	35.34	36	140/141, 392/393; 168/169, 364/365; 196/197, 336/337
63	11,23-; 13,19-; 13,21-; 15,19-Dimethylpentatriacontane[c]	35.60	37	168/169, 224/225, 351, 379; 196/197, 252/253, 295, 351; 196/197, 323, 351; 224/225, 252/253, 295, 323
64	Heptatriacontene	36.70	37:1	518
65	11,15,23-; 13,17,23-Trimethylpentatriacontane[c]	36.78	38	168/169, 196/197, 239, 323, 365, 393; 196/197, 267, 295, 365
66	Heptatriacontane	37.00	37	520
67	9-; 11-; 13-Methylheptatriacontane[c]	37.30	38	140/141, 406/407; 168/169, 392/393; 196/197, 364/365
68	13,21-; 15,21-Dimethylheptatriacontane[c]	37.51	39	196/197, 252/253, 323, 379; 224/225, 252/253, 323, 351
69	13,17,21-; 13,17,23-Trimethylheptatriacontane	37.90	40	196/197, 252/253, 267, 323, 337, 393 196/197, 224/225, 267, 323, 365, 393
70	11-; 12-; 13-Methyloctatriacontane[c]	38.30	39	168/169, 406/407; 182/183, 392/393; 196/197, 378/379
71	9-; 11-; 13-; 15-Methylnonatriacontane[c]	39.30	40	140/141, 448/449; 168/169, 420/421; 196/197, 392/393; 224/225, 364/365

TABLE 1. Continued

Peak[a]	Hydrocarbon	ECL[b]	CN[b]	Diagnostic MS ions
72	13,17-; 13,21-; 15,19-Dimethylnonatriacontane[c]	39.60	41	196/197, 336/337, 267, 407; 196/197, 280/281, 323, 407; 224/225, 308/309, 295, 379
73	13,17,23-; 13,17,25-Trimethylnonatriacontane[c]	39.90	42	196/197, 252/253, 267, 365, 351, 421; 196/197, 224/225, 267, 351, 393, 421
74	11-; 13-; 15-Methylhentetracontane[c]	41.30	42	168/169, 448/449; 196/197, 420/421; 224/225, 392/393
75	13,17-; 13,21-Dimethylhentetracontane	41.60	43	196/197, 364/365, 267, 435 196/197, 308/309, 323, 435
76	13,17,21-Trimethylhentetracontane	41.90	44	196/197, 308/309, 267, 337, 379, 449
77	11-; 13-; 15-Methyltritetracontane[c]	43.30	44	168/169, 476/477; 196/197, 448/449; 224/225, 420/421
78	13,17-Dimethyltritetracontane	43.60	45	196/197, 392/393, 267, 463

[a] Peak numbers refer to peaks identified in Figures 1–4.
[b] ECL = equivalent chain length, CN = carbon number.
[c] An isomeric mixture or two or more components coelute in this peak.

TABLE 2. HYDROCARBONS IDENTIFIED FROM EIGHT SPECIES OF EIGHT SPECIES OF *Conophthorus*[A]

Peak No.	Hydrocarbon	ECL	*Conophthorus* species[b]							
			Coni	*Cpon*	*Ccem*	*Cedu*	*Crad*	*Ccon*	*Cres*	*Cbun*
1	2-Methyleicosane	20.70	o	o	o	o	o	+	o	o
2	*n*-Heneicosane	21.00	o	o	o	o	o	tr	o	o
3	*n*-Docosane	22.00	tr	+	tr	tr	tr	+	tr	tr
4	2- or 4-; 3-Methyldocosane[c]	22.76	o	tr	o	o	o	o	o	o
5	Tricosene	22.76	o	tr	o	o	o	o	o	o
6	*n*-Tricosane	23.00	+++	+++	++	++	+++	+++	+++	+++
7	9-; 11-; 13-Methyltricosane[c]	23.39	tr	o	tr	o	o	o	o	o
8	Tetracosene	23.70	o	tr	o	o	o	o	tr	tr
9	3-Methyltricosane	23.73	tr	tr	tr	o	tr	o	o	o
10	*n*-Tetracosane	24.00	+	++	+	tr	++	+	+	+
11	2- or 4-Methyltetracosane	24.63	o	tr	o	o	o	o	o	o
12	Pentacosadiene	24.69	o	tr	o	o	++	tr	++	++
13	Pentacosene	24.70	+++	+++	+	+	+++	+++	+++	+++
14	*n*-Pentacosane	25.00	+++	+++	+++	+++	+++	+++	+++	+++
15	9-; 11-; 13-Methylpentacosane[c]	25.38	+	tr	+	+	tr	++	+	+
16	5-Methylpentacosane	25.50	tr	tr	tr	o	o	o	o	o
17	11,15-Dimethylpentacosane	25.60	o	o	tr	tr	o	o	o	o
18	2- or 4-Methylpentacosane	25.70	o	o	o	o	o	tr	o	o
19	Hexacosene + 3-methylpentacosane[c]	25.73	tr	tr	o	o	o	o	o	o
			tr	tr	tr	tr	tr	+	+	+
20	*n*-Hexacosane	26.00	++	++	++	++	++	++	++	++
21	12-; 13-Methylhexacosane	26.34	tr	tr	tr	tr	o	++	o	o
22	11,15-; 13,17-Dimethylhexacosane[c]	26.60	o	o	+	o	o	o	o	o
23	2- or 4-Methylhexacosane +	26.63	o	o	o	o	o	++	o	o
	heptacosadiene[c]		o	tr	o	o	o	++	tr	tr
24	Heptacosene	26.70	+	+	tr	++	+	+++	++	++

TABLE 2. Continued

Peak No.	Hydrocarbon	ECL	*Conophthorus species*[b]							
			Coni	*Cpon*	*Ccem*	*Cedu*	*Crad*	*Ccon*	*Cres*	*Cban*
25	3-Methylhexacosane	26.79	o	tr	o	o	o	o	o	o
26	*n*-Heptacosane	27.00	+++	+++	+++	+++	+++	+++	+++	+++
27	7-; 9-; 11-; 13-Methylheptacosane[c]	27.32	+	++	++	++	tr	+++	+	+
28	9-Methylheptacosane	27.40	o	o	o	o	o	+	o	o
29	7-Methylheptacosane	27.42	o	o	o	o	o	+	o	o
30	5-Methylheptacosane	27.50	tr	tr	o	o	o	+	o	o
31	9,13-Dimethylheptacosane + 9,15-dimethylheptacosane + 11,15-dimethylheptacosane[c]	27.63	tr	o	o	o	o	o	o	o
			o	o	+++	+++	o	o	o	o
			tr	o	+++	+++	o	o	o	o
32	3-Methylheptacosane	27.74	tr	tr	o	o	tr	x	+	+
33	*n*-Octacosane	28.00	+	+	++	+	tr	x	tr	tr
34	9-; 11-; 13-; 15-Methyloctacosane	28.32	o	tr	tr	tr	o	+	o	o
35	11,15-; 13,17-Dimethyloctacosane[c]	28.62	o	o	+	tr	o	o	o	o
36	3-Methyloctacosane	28.70	o	tr	o	o	o	o	o	o
37	*n*-Nonacosane	29.00	++	++	+++	++	+	x	+	+
38	9-; 11-; 13-; 15-Methylnonacosane[c]	29.32	tr[d]	tr	++	++	o	++[e]	o	o
39	5-Methylnonacosane	29.50	o	tr	o	o	o	o	o	o
40	11,15-; 13,17-Dimethylnonacosane[c]	29.61	tr	o	+++	+++	o	o	o	o
41	3-Methylnonacosane	29.70	o	tr	o	o	o	o	o	o
42	13,17,21-Trimethylnonacosane	29.90	o	o	+	tr	o	o	o	o
43	*n*-Triacontane	30.00	+	tr	tr	tr	o	o	o	o
44	3,7-Dimethylnonacosane	30.10	o	tr[g]	x	x	o	o	o	o
45	11,15-; 13,17-Dimethyltriacontane[c]	30.60	o	o	tr	x	o	o	o	o
46	*n*-Hentriacontane	31.00	++	+	++	++	o	o	++	++
47	7-, 9-, 11-, 13-, 15-Methylhentriacontane	31.40	tr	o	+	+	o	o	o	o

48	11,15-; 13,17-Dimethylhentriacontane	31.64	+	o	++	++	tr[f]	o	o	o
49	Docotriacontene + 3-methyltriacontane[c]	31.71	o	tr	o	o	o	o	o	o
			o	tr	o	o	o	o	o	o
50	11,15,19-Trimethylhentriacontane	31.90	+	o	o	o	o	o	o	o
51	*n*-Docotriacontane	32.00	tr	o	o	o	o	o	o	o
52	3,7-Dimethylhentriacontane	32.10	tr	+[g]	o	o	o	o	o	o
53	Tritriacontene	32.70	o	tr	o	tr	o	o	o	o
54	*n*-Tritriacontane	33.00	+	tr	tr	tr	o	o	o	o
55	9-; 11-; 13-; 15-Methyltritriacontane[c]	33.30	+	+	++	+	tr	x	o	o
56	11,15-; 11,19-; 11,21-; 13,19-Dimethyltritriacontane[c]	33.57	o	tr	o	o	o	x	o	o
	11,19-; 13,19-dimethyltritriacontane[c]		++	o	o	o	o	o	o	o
	13,17-Dimethyltritriacontane		o	o	tr	+	o	++	o	o
57	11,15,21-; 13,17,23-Trimethyltritriacontane[c]	33.86	++	+	o	o	o	o	o	o
	13,17,21-Trimethyltritriacontane		o	o	tr	++	o	++	o	o
58	3,7-Dimethyltritriacontane	34.08	tr	tr[g]	o	o	o	o	o	o
59	11-; 12-; 13-Methyltetratriacontane[c]	34.30	o	tr	tr	x	o	o	o	o
60	Pentatriacontene	34.70	o	tr	o	o	o	o	++	++
61	*n*-Pentatriacontane	35.00	++	o	x	x	o	o	o	o
62	9-; 11-; 13-Methylpentatriacontane[c]	35.31	++	++	++	++	++	o	+	tr
63	11,23-; 13,21-; 15,19-Dimethylpentatriacontane[c]	35.60	++	o	o	o	o	o	o	o
	11,23-; 15,19-Dimethylpentatriacontane[c]		o	+	o	o	o	o	o	o
	13,19-; 13,21-Dimethylpentatriacontane[c]		o	o	o	o	++	o	o	o
	13,21-; 15,19-Dimethylpentatriacontane[c]		o	o	o	o	o	+++	o	o
	13,19-Dimethylpentatriacontane		o	o	tr	+	o	o	o	o
64	11,15,23-Trimethylpentatriacontane	35.78	++	++	o	o	o	o	o	o
	13,17,23-Trimethylpentatriacontane		++	++	tr	tr	+	++	++	++
65	Heptatriacontene	36.70	o	o	o	o	o	o	++	++
66	Heptatriacontane	37.00	+	o	o	o	o	o	o	o
67	9-; 11-; 13-Methylheptatriacontane[c]	37.30	++	++	++	++	++	x	++	++

TABLE 2. Continued

Peak No.	Hydrocarbon	ECL	*Conophthorus species*[b]							
			Coni	*Cpon*	*Ccem*	*Cedu*	*Crad*	*Ccon*	*Cres*	*Cban*
68	13,21-; 15,21-Dimethylheptatriacontane[c]	37.59	++	+	o	tr[h]	+++	+++	+	+
69	13,17,21-Trimethylheptatriacontane[c]	37.90	+	++	o	o	++	o	++	++
	13,17,23-Trimethylheptatriacontane		o	++	o	o	++	x	o	o
70	11-; 12-; 13-Methyloctatriacontane[c]	38.30	tr	tr	tr	o	o	o	o	o
71	9-; 11-; 13-; 15-Methylnonatriacontane[c]	39.30	++	++	++	+++	++	o	++	++
72	13,17-; 13,21-; 15,19-Dimethylnonatriacontane[c]	39.60	++	+	++[h]	++[h]	+++[h]	o	x	x
73	13,17,23-; 13,17,25-Trimethylnonatriacontane[c]	39.90	++	+	o	o	++	o	++	++
74	11-; 13-; 15-Methylhentetracontane[c]	41.30	++	+	++	++	x	o	+	+
75	13,17-Dimethylhentetracontane	41.60	++	+	o	o	x	o	x	x
	13,21-Dimethylhentetracontane		+	o	+	+	o	o	x	x
76	13,17,21-Trimethylhentetracontane	41.90	++	+	o	o	x	o	x	x
77	11-; 13-; 15-Methyltritetracontane[c]	43.30	+	o	++	++	o	o	o	o
78	13,17-Dimethyltritetracontane	43.60	++	o	+	+	o	o	o	o

[a] Hydrocarbon identification by GC-MS (see Table 1 and Figures 1–4). A triple + indicates ≥5.0%, ++ from 1.0 to 5.0%, and + from 0.5 to 1.0% of the total hydrocarbon component. Some trace (tr) components appear infrequently or consistently in very small quantities (<0.5% of the total). A zero indicates the hydrocarbon was never identified for the species. Compounds with an x were not identified; mass spectra were insuffient to make positive identification.

[b] Coni = *Conophthorus conicolens*, Cpon = *C. ponderosae*, Ccem = *C. cembroides*, Cedu = *C. edulis*, Ccon = *C. coniperda*, Crad = *C. radiatae*, Cres = *C. resinosae*, Cban = *C. banksianae*.

[c] An isomeric mixture. These components coelute in this peak.

[d] The 9-methyl isomer is the only component present.

[e] The 13- and 15-monomethylalkanes coelute in this peak.

[f] Only the 13,17-dimethyl isomer was identified.

[g] The 3,7-dimethyl isomers were found only in adults from sugar pine cones.

[h] The 13,21-dimethyl isomer was the only component identified.

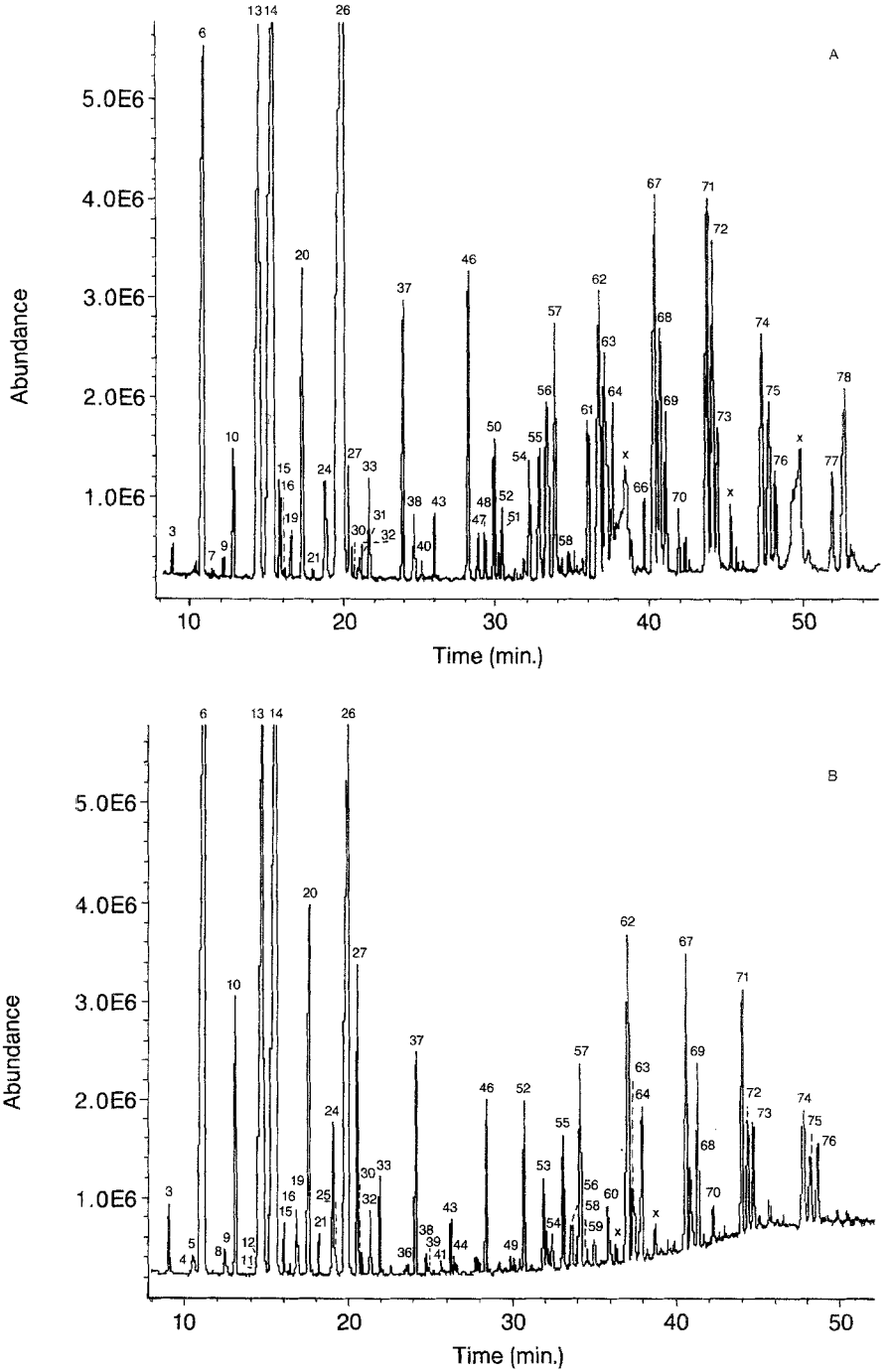

FIG. 1. Total ion chromatograms of cuticular hydrocarbons from: (A) *Conophthorus conicolens* collected from Chihuahua pine from the Chiricahua Mountains in southeastern Arizona, and (B) *C. ponderosae* collected from sugar pine from the Eldorado National Forest, California. Numbers identify peaks whose compositions are listed in Table 1. Compounds marked with an x were not identified.

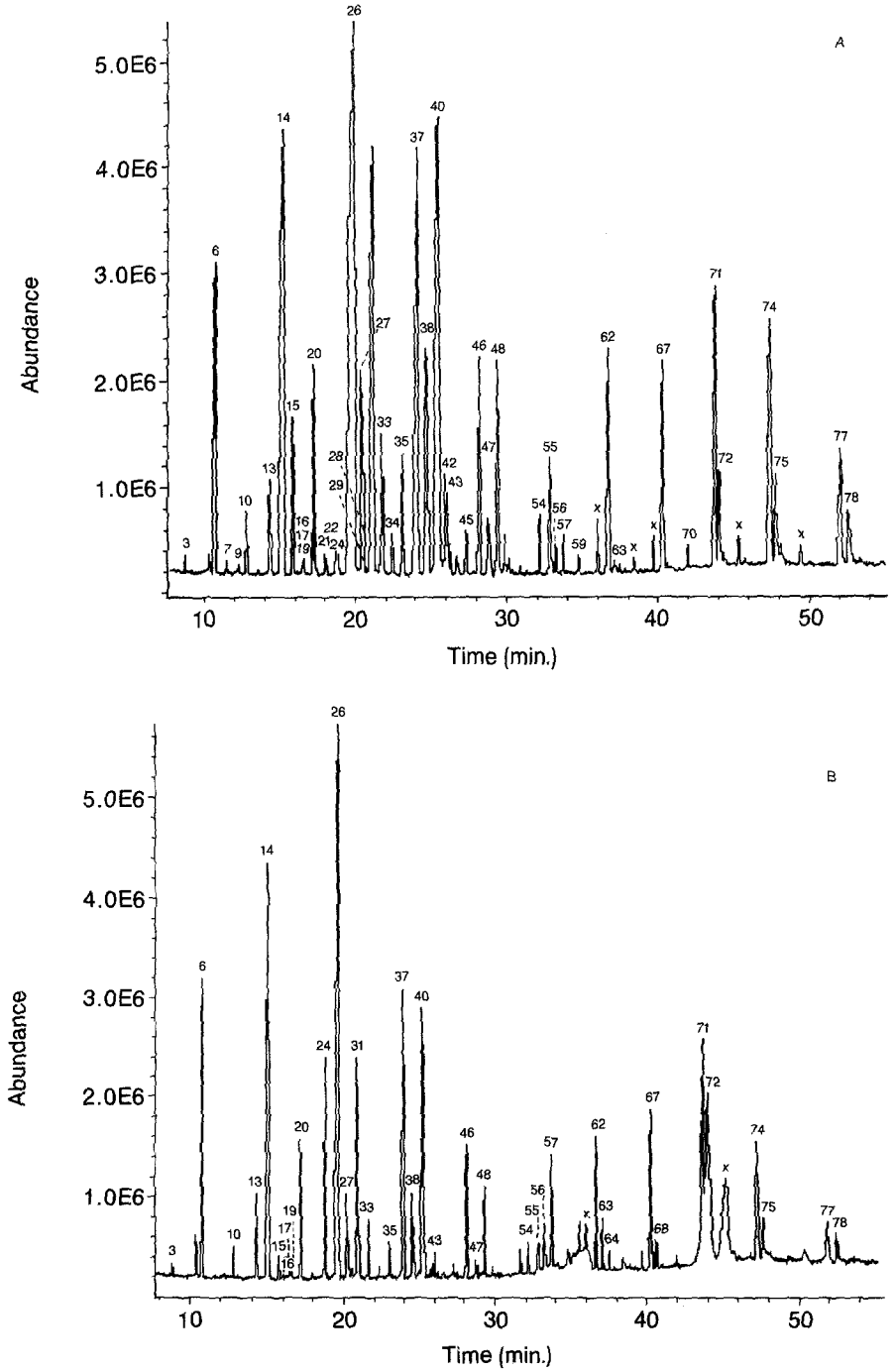

FIG. 2. Total ion chromatograms of cuticular hydrocarbons from: (A) *Conophthorus cembroides* collected from Mexican pinyon from the Chiricahua Mountains in southeastern Arizona, and (B) *C. edulis* collected from pinyon pine from Prescott, Arizona. Numbers identify peaks whose compositions are listed in Table 1. Compounds marked with an x were not identified.

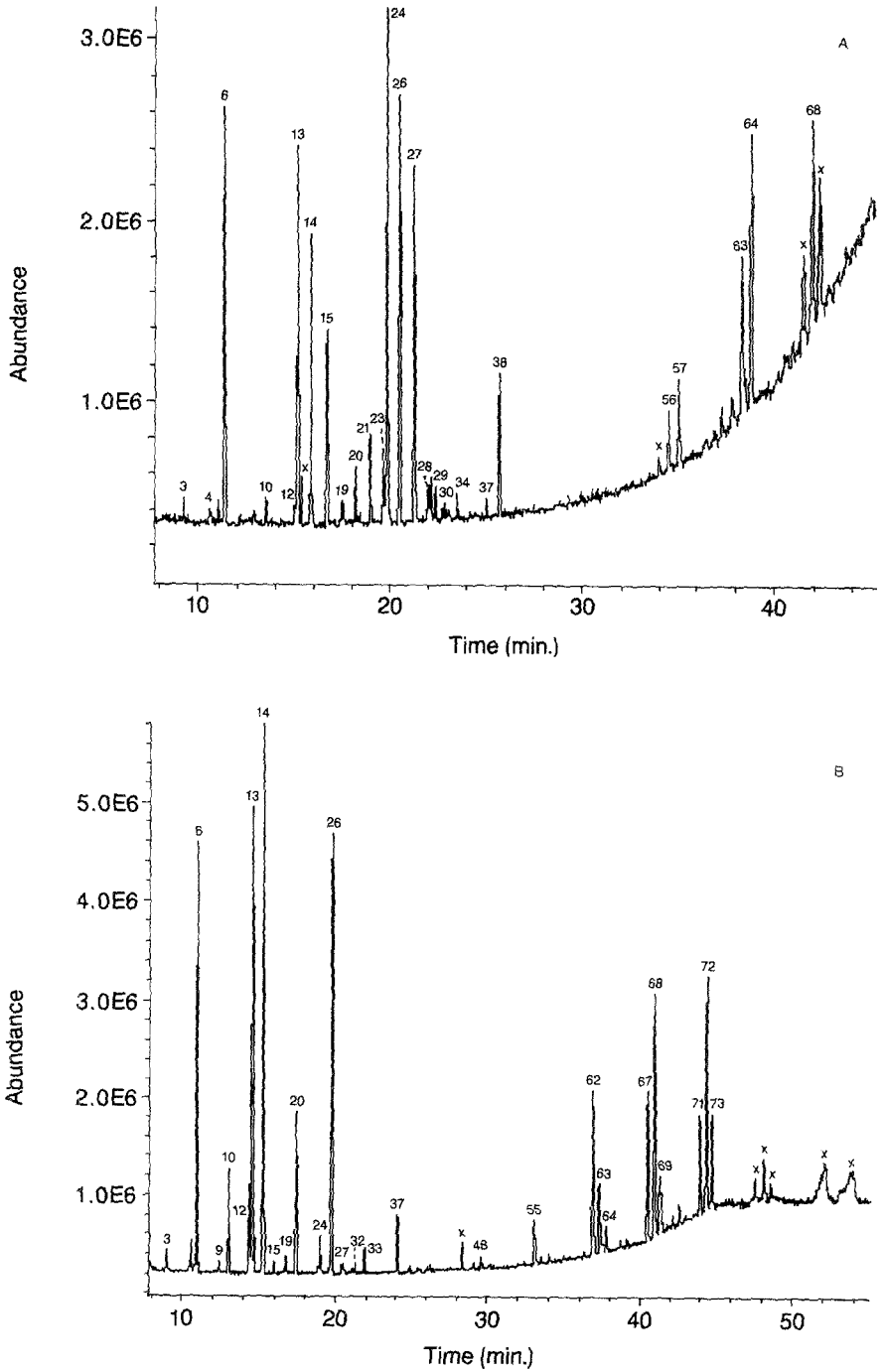

FIG. 3. Total ion chromatograms of cuticular hydrocarbons from: (A) *Conophthorus coniperda* collected from eastern white pine from Murphy, North Carolina, and (B) *C. radiatae* collected from Monterey pine from Albany, California. Numbers identify peaks whose compositions are listed in Table 1. Compounds marked with an x were not identified.

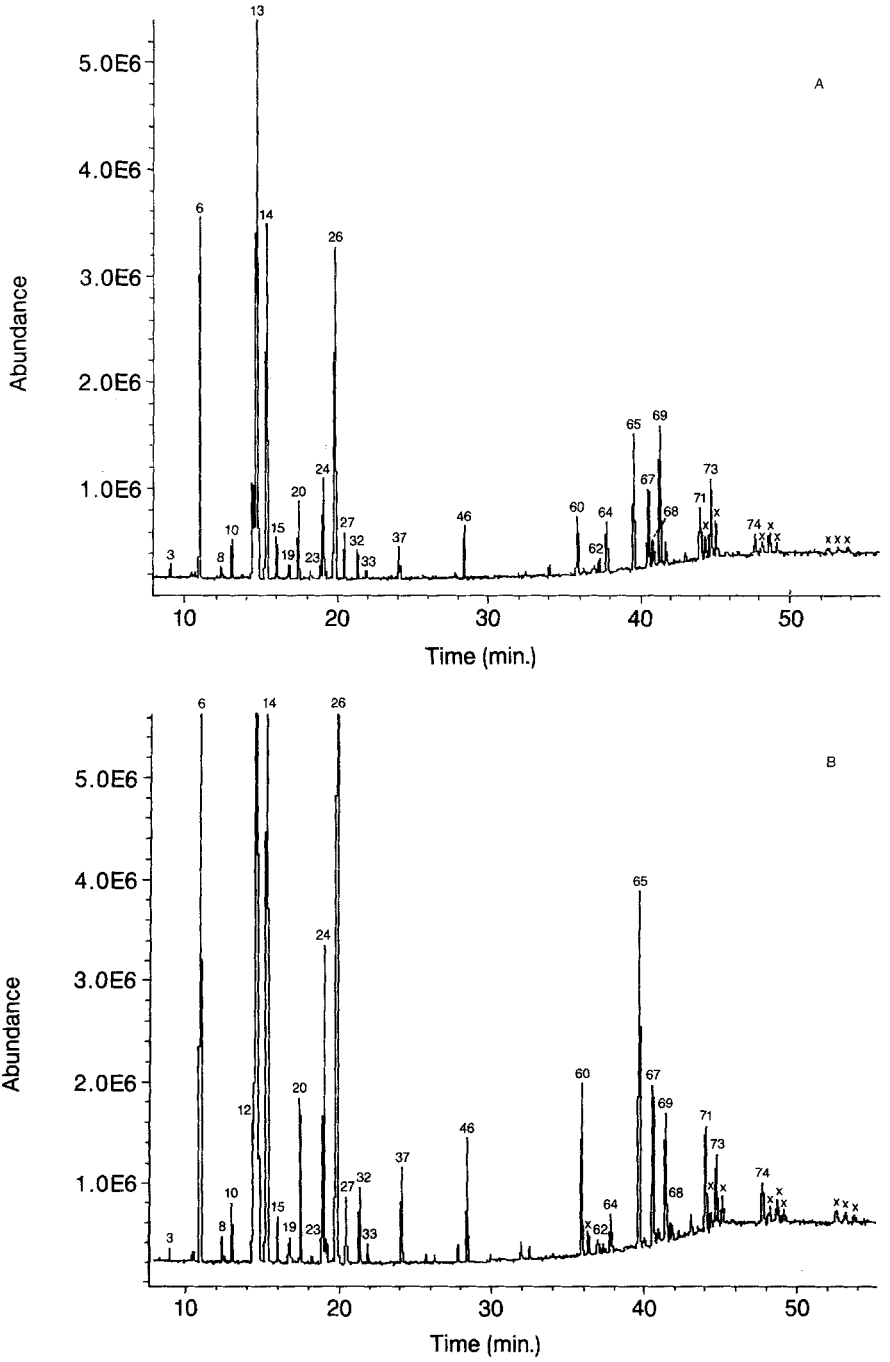

FIG. 4. Total ion chromatograms of cuticular hydrocarbons from: (A) *Conophthorus resinosae* collected from red pine from Little Rapids, Ontario, Canada, and (B) *C. banksianae* collected from jack pine twigs from Aubrey Falls, Ontario, Canada. Numbers identify peaks whose compositions are listed in Table 1. Compounds marked with an x were not identified.

Significant (>0.5% total hydrocarbon) internally branched monomethylalkanes occur in these *Conophthorus* species as complex isomeric mixtures. The mass spectra of peaks with ECL of approximately 0.7 before the corresponding C_n have been identified as having methyl branches on odd-numbered carbons (9 through 15). They have strong ions at *m/z* 140:141, 168:169, 196:197 and 224:225, indicating alpha cleavage internal to the branches at 9, 11, 13, and 15 carbons. *C. conicolens*, *C. cembroides*, and *C. edulis* produce the only homologous series of isomeric mixtures that includes all 11- and 13-(Me) C_n compounds having odd-numbered parent chains from 11-; 13-(Me) C_{25} to 11-; 13(Me) C_{34}. The 9-(Me) C_n isomers are present from 9-(Me) C_{25} to 9-(Me) C_{35}. The only exception is *C. conicolens*, which produces only 9-(Me) C_{29} and adds the 15-(Me) C_n isomers at 15-(Me) C_{33}, 15-(Me) C_{39}, 15-(Me) C_{41}, and 15-(Me) C_{43}.

There were only five individual or isomeric mixtures of internally branched monomethylalkanes identified with even-numbered parent chains (Tables 1 and 2). *C. ponderosae* and *C. coniperda* both produce 12-; 13-(Me) C_{26}. 15-(Me) C_{28} is found in *C. coniperda* and 11-; 12-; 13-(Me) C_{38} in *C. conicolens*. Traces of 11-; 12-(Me) and 13-(Me) C_{34} are found in *C. ponderosae* and *C. cembroides*. Small amounts of single-component 5- and 7-monomethyalkanes are present in *C. ponderosae* and *C. coniperda*. Trace amounts of 5-(Me) C_{25}, 5-(Me) C_{27}, and 5-(Me) C_{29} were identified in all of the *C. ponderosae* populations. *C. coniperda* is the only species with a single-component 7-(Me) C_n, identified as 7-(Me) C_{27}.

Dimethylalkanes in insects generally have methyl branches on odd-numbered carbons separated by an odd number of carbons (Blomquist et al., 1987). All of the dimethylalkanes produced by these species of *Conophthorus* fit this pattern. The majority of the methyl branches are separated by three carbons. They include the 3,7-$(Me)_2$ C_n (3,7-dimethylalkanes) (*C. ponderosae*, sugar pine, and *C. conicolens*), the 11,15- and 13,17-$(Me)_2$ C_n (*C. conicolens*, *C. ponderosae*, *C. cembroides*, *C. edulis*, *C. radiatae*), 15,19-$(Me)_2$ C_{35} (*C. conicolens*, *C. ponderosae*, *C. coniperda*) and 9,13-$(Me)_2$ C_{27} in *C. conicolens*. The remainder of the dimethylalkanes have methyl branches separated by 5, 7, 9, and 11 carbons. Dimethylalkanes with methyl branches separated by five carbons include 9,15-$(Me)_2$ C_{27} (*C. cembroides*, *C. edulis*), 13,19-$(Me)_2$ C_n (*C. conicolens*, *C. ponderosae*, *C. cembroides*, *C. edulis*, and *C. radiatae*), and 15,21-$(Me)_2$ C_{37} in all species except *C. cembroides* and *C. edulis*. All species include representatives of dimethylalkanes separated by 7 carbons: 11,19-$(Me)_2$ C_{33}; 13,21-$(Me)_2$ C_{35}; 13,21-$(Me)_2$ C_{37}, and 13,21-$(Me)_2$ C_{41}. The only component with methyl branches separated by nine carbons was 11,21-$(Me)_2$ C_{33} in an isomeric mixture in *C. ponderosae*. The one dimethylalkane with 11 carbons separating methyl branches was 11,23-$(Me)_2$ C_{35} in *C. conicolens* and *C. ponderosae*.

We identified isomeric mixtures of species specific dimethylalkanes in *C. ponderosae*, *C. conicolens*, *C. cembroides*, and *C. edulis* (Table 2). Dimethylalkanes that eluted about 0.9 carbon units in front of the corresponding *n*-

alkane were identified as 3,7-$(Me)_2$ C_n isomers. An example of the mass spectrum of this type of component is interpreted as 3,7-$(Me)_2$ C_{31} (Figure 5A). The $(M-29)^+$ and $(M-57)^+$ ions at *m/z* 435 and 407 localize one methyl branch on the 3-position. The ion fragments at *m/z* 364/365 and 127 show that the other methyl branch is on carbon 7. The absence of other diagnostic ions indicates that this isomer is the only prominent one in this peak. In *C. ponderosae* three 3,7-$(Me)_2$ C_n (peaks 44, 52, and 58) were unique to beetles infesting sugar pine cones. Thus far we have not found these components in adults infesting ponderosa pine or western white pine cones.

Dimethylalkanes with both methyl branches positioned internally elute ca. 1.4 ECL in front of the corresponding *n*-alkane. Unique species-specific dimethylalkanes with such ECLs are present in *C. conicolens*, *C. cembroides*, and *C. edulis* (Table 2). The hydrocarbon mixtures of *C. cembroides* and *C. edulis* contain significant amounts of 9,15- and 11,15-$(Me)_2$ C_{27} (peak 31); 11,15- and 13,17-$(Me)_2$ C_{29} (peak 40), and 11,15- and 13,17-$(Me)_2$ C_{31} (peak 48). An example of the mass spectrum of this type of dimethylalkane was interpreted as 11,15-$(Me)_2$ C_{27} (Figure 5B). The fragments at *m/z* 168/169 and 196/197, where the even-to-odd ratio is greater than one, arise from cleavage internal to each of the carbons bearing methyl branches. The ion fragment at *m/z* 239 and 267, in which the odd-numbered ions predominate, arise from cleavage external to the two branching methyl groups. The mass spectra of the other internally branched dimethylalkanes were interpreted in a similar manner (Tables 1 and 2).

Homologous series of high-molecular-weight trimethylalkanes are found in all eight species of *Conophthorus*. Each component has an ECL of approximately 2.1 less than the corresponding *n*-alkane. *C. cembroides* and *C. edulis* contain fewer trimethylalkanes than the other six species. *C. radiatae*, *C. coniperda*, *C. resinosae*, and *C. banksianae* appear to only produce trimethylalkanes with parent carbon chains of 35, 37, and 39. *C. conicolens* and *C. ponderosae* produce isomeric mixtures of trimethylalkanes from parent carbon chains 33–41. We identified a homologous series of 13,17,23-$(Me)_3$ C_n (13,17,23-trimethylalkanes) in *C. conicolens* and *C. ponderosae* starting with 13,17,23-$(Me)_3$ C_{33} (Figure 6A). The component 13,17,23-$(Me)_3$ C_{35} (peak 64) is found in all *Conophthorus* species characterized in this paper. The significant even-mass ions *m/z* 196 : 197, with an even-to-odd number ratio of about 1, are interpreted as arising from a cleavage internal to the methyl branches at either end of the molecule. The ions at *m/z* 267, 295, and 365 arise from cleavage external to the methyl branch on positions 13 and 23 and on both sides of the methyl branch on position 17. The odd-to-even ratio for these ions is greater than 1, as secondary carbonium ions containing two of three methyl branches supress formation of even-mass ions (Blomquist et al. 1987). *C. conicolens* and *C. ponderosae* clearly have the most complex series of trimethylalkanes. In these species, we have identified a unique trimethylalkane (peak 73) that occurs

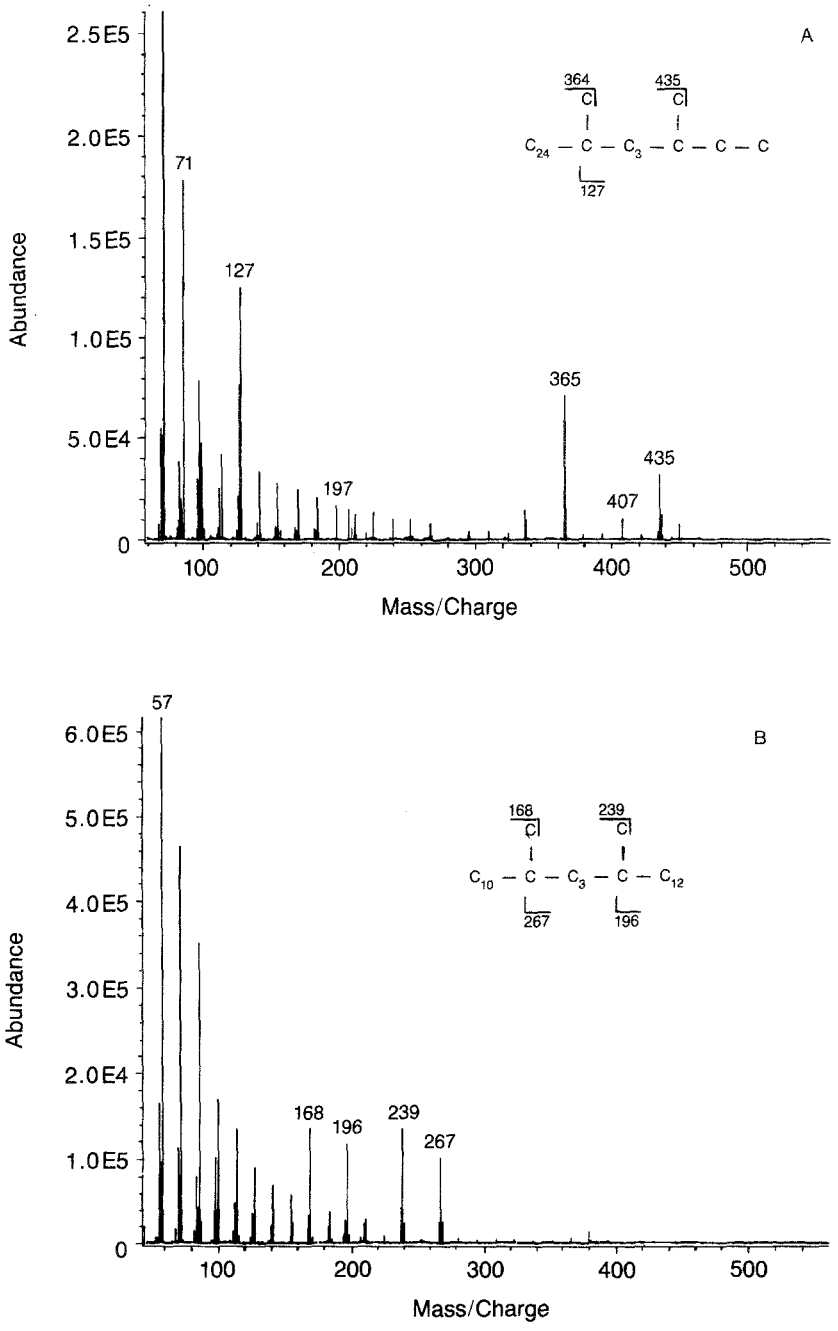

FIG. 5. EI mass spectra of (A) peak 52, Figure 1B, identified as 3,7-dimethylhentriacontane, and (B) peak 31, Figure 2A, identified as 11,15-dimethylheptacosane.

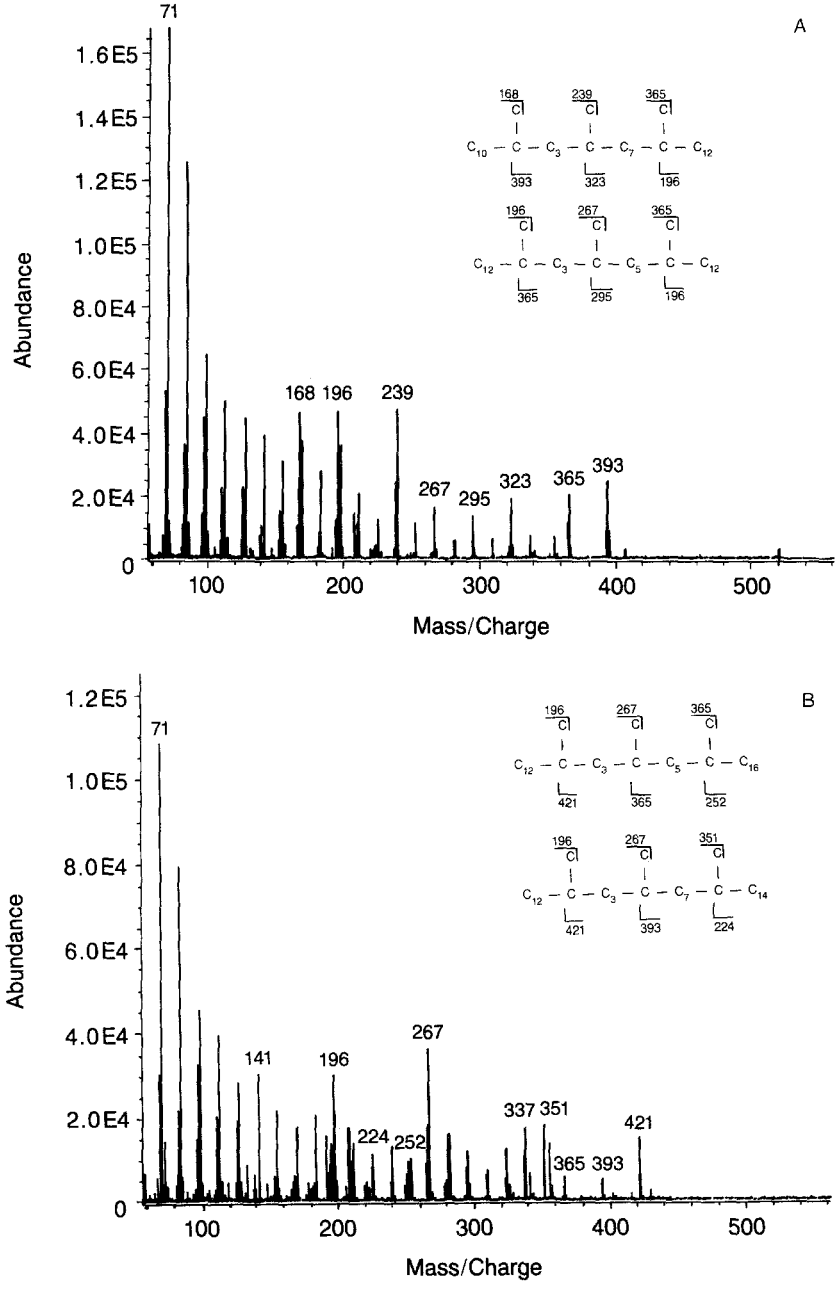

FIG. 6. EI mass spectrum of peak 65, Figure 1A and B, identified as an isomeric mixture of 11,15,23- and 13,17,23-trimethylpentatriacontane, and (B) peak 73, Figure 1B, identified as 13,17,25-trimethylnonatriacontane.

in an isomeric mixture whose mass spectrum is interpreted as 13,17,25-$(Me)_3$ C_{39} (Figure 6B). This component has even-mass ions at *m/z* 196/197 and 224/225 and significant odd ions at *m/z* 267, 351, 393 and 421.

DISCUSSION

The cuticular hydrocarbons of *Conophthorus*, as is the case for many insect species (Blomquist et al., 1987), consist of a complex mixture of unsaturated and straight-chain and methyl-branched, saturated components. The relatively large number of components, ease of analysis, and species-specific compositions for many insects make them attractive characters for use in chemotaxonomy (Carlson and Bolten, 1984; Gastner and Nation, 1986; Haverty et al., 1988; Howard et al., 1988; Lockey, 1982; Vander Meer, 1986).

The cuticular hydrocarbons, particularly the unsaturated and methyl-branched components, are synthesized by the insect (Blomquist et al., 1987). A small portion of the *n*-alkanes in several species can arise from the diet (Blomquist and Jackson, 1973; Nelson et al., 1971), but unsaturated and methyl-branched hydrocarbons are rare in plants. Thus, the prevalence of unsaturated and methyl-branched components in *Conophthorus*, while expected, is particularly useful.

Conophthorus is a genus in which many of the species are difficult to separate on the basis of morphological characters alone. In this report we have identified all the major cuticular hydrocarbons of eight of the 15 described species of *Conophthorus* (Flores and Bright, 1987; Wood, 1982). We hope to develop a new suite of characters for the entire genus, similar to our studies with the dampwood termites, *Zootermopsis* (Haverty et al., 1988; Haverty and Thorne, 1989; Thorne and Haverty, 1989). The cuticular waxes of these species of *Conophthorus* contain many diagnostic hydrocarbons, which are useful for separation of these species.

Reports of trimethylalkanes in cuticular lipids of Coleoptera are rare, although they have been reported from other groups of insects (Blomquist et al., 1987). So far, trimethylalkanes have been positively identified only in *Cylindrinotus laevioctostriatus* (Goeze) and *Phylan gibbus* (Fabricius) in the Tenebrionidae (Lockey, 1981). Trimethyalkanes may be ubiquitous in all species of *Conophthorus*. Each of the eight species we examined in this study produces significant amounts of trimethylalkanes after C_{30}. The spacing between methyl groups in other Coleoptera has been a [3,3] sequence. We have identified trimethylalkanes with [3,5] and [3,7] spacing. The [3,5] spacing of 11,15,21- and 13,17,23-$(Me)_3$ C_n isomers is the most common in all of these species of *Conophthorus*. This spacing has been identified so far only in the components 11,15,21- and 13,17,23-$(Me)_3$ C_{35} and 11,15,21- and 13,17,23-$(Me)_3$ C_{37} in the female tsetse fly, *Glossina pallidipes* (Austen) (Nelson and

Carlson, 1986). We have identified the components 11,15,21- and 13,17,23-$(Me)_3$ C_{33} and 13,17,25-$(Me)_3$ C_{39} in several species of *Conophthorus*, in addition to those trimethylalkanes reported in *G. pallidipes*. This appears to be the first report of a trimethylalkane with a [3,7] spacing sequence between methyl branches.

The composition of the cuticular wax components in three populations of the polyphagous species *Conophthorus ponderosae* from three different hosts is one of the most complex. With one possible exception, the hydrocarbon blends are qualitatively the same in all three of the populations we examined; the exceptions are the 3,7-$(Me)_2$ C_n in the populations from sugar pine. It may be possible that *C. ponderosae* in ponderosae pine and western white pine produce these dimethylalkanes in significantly lower quantities. Future analyses with more adults from additional populatations may resolve their presence or absence. If one agrees that hydrocarbon profiles are species specific (Haverty et al., 1988; Howard and Blomquist, 1982; Howard et al., 1982; Vander Meer, 1986), we would infer that two populations evaluated here (from ponderosa pine and western white pine) are of the same species or at least very closely related.

The status of *C. cembroides* and *C. edulis* as distinct, separate species is in question (S.L. Wood, personal communication). Our analyses of their hydrocarbon components suggest that they are closely related. The only difference in hydrocarbon patterns is the replacement of a dimethylalkane at ECL = 26.6 with an alkene (ECL = 26.7) in *C. cemroides*. Extensive sampling of *Conophthorus* from the nut pines in the southwestern United States and northern Mexico should clarify the relationship of these two similar species.

The hydrocarbon patterns of *C. resinosae* and *C. banksianae* are qualitatively identical; no unique hydrocarbon components were found between these two species from many populations in Canada and the United States. They do possess several components that easily separate them from the other six species discussed in this paper. This evidence supports the suspicion that *C. banksianae* is not a valid species (S.L. Wood and P. de Groot, personal communications). Further elucidation with biological and genetic studies will be necessary to resolve this problem.

Future studies will involve further characterization of the cuticular hydrocarbons of additional species of *Conophthorus* and quantitative evaluation of intra- and interspecific variation. It is hoped these studies will clarify and/or validate the taxonomy of this genus, especially the polyphagous species, *C. ponderosae* and the *C. edulis* complex. Once we have characterized the cuticular hydrocarbons of all of the extant species of *Conophthorus*, we will develop a dichotomous key to all species. To be useful for dichotomous keys, hydrocarbons should be abundant, not minor, components (at least 1%, but preferably $>5\%$ of the total hydrocarbon mixture). They should be unique or present in only a few of the species, or conversely, they should be common in most of

the species yet completely absent, rare, or present in insignificant quantities in one or a few. Furthermore, they should have a unique elution time so that they do not coelute with another hydrocarbon in the same species, nor should they elute at a time similar to a different hydrocarbon in a different species.

Acknowledgments—We thank Paul Tilden, Gary DeBarr, Peter deGroot, and Patrick Shea for their help in collecting *Conophthorus* specimens; and Gary DeBarr, Ralph Howard, and Peter de Groot for providing helpful reviews of early versions of the manuscript. This work was supported, in part, by a grant to M.I.H., M.P., and G.J.B. from the Internal Competitive Grant Program, Pacific Southwest Forest and Range Experiment Station.

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STRONG REPELLENCY OF THE ROOT KNOT NEMATODE, *Meloidogyne incognita* BY SPECIFIC INORGANIC IONS

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Abstract—Simple inorganic salts of the ions K^+, NH_4^+, Cs^+, NO_3^-, and Cl^- are strongly repellent to infective second-stage larvae of the root knot nematode, *Meloidogyne incognita*. Some of these salts are known to be beneficial to plant growth. The results suggest a new means of plant protection.

Key Words—Chemotaxis, repellents, *Meloidogyne incognita*, root knot nematode, inorganic ions, salts, gradients.

INTRODUCTION

Attraction of parasitic nematodes by plant roots may be the key to host recognition, specificity, and subsequent infection (Dusenbery, 1987; Steiner, 1925). On the other hand, environmentally tolerable repellents offer an alternative to pesticides in the protection of plants from these root parasites.

We have recently described a quantitative bioassay for the attraction and repulsion of plant parasitic nematodes by chemical substances (Castro et al., 1989). The method was illustrated with infective second-stage larvae of *Meloidogyne incognita* (MiJ_2) and chemotactic fractions isolated from cucumber roots. In the course of this work we have found that fertilized cucumber seedlings contained an additional repellent fraction. Examination of the fertilizer constituents prompted a scan of the response of MiJ_2 to a variety of inorganic salts. In this work we describe the surprisingly strong repellency of simple inorganic ions to this parasite. Our results suggest that salts beneficial to plant nutrition,

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suitably applied, may be used to shield roots from nematode attack. They suggest a new means of plant protection.

METHODS AND MATERIALS

^{14}C-Labeled glycerine, glycine, and sodium acetate were commercial samples (New England Nuclear, Dupont) and were used without purification. Specific activities were UL labeled glycerine 9.1 mCi/mmol, [1-^{14}C]glycine 4.5 mCi/mmol, and [1-^{14}C]sodium acetate 1.8 mCi/mmole. HFeEDTA · $2H_2O$ was prepared by literature procedures (Garcia Basalotte et al., 1986) and recrystallized twice from water–acetone before use. Monosodium and potassium salts were obtained from it by reaction with stoichiometric amounts of the corresponding carbonates. The resulting monohydrate complexes were recrystallized from water–acetone. All other salts used in this work were purchased as analytical reagent-grade substances.

Bioassay. The essence of our bioassay is to effectively restrict nematode movement to one dimension. Narrow 0.8% agarose tracks are employed. Precise gradients of test substance can be established, and the population of nematodes along the track can be monitored with time. The test substance is placed at the end of the track at fixed concentration. After a suitable time (ΔT), the nematodes (100–150 larvae) are inoculated into the center of the track and the population distribution is allowed to "develop." Employing the average number of animals in each 0.5-cm section of track from replicate runs (N), and the distance of that section from the center (D), we have defined an attractant (A) repellent (R) ratio at time t as:

$$(A/R)_t = \frac{\sum (N)(D)_{\text{toward}}}{\sum (N)(D)_{\text{away}}}$$

The sum $(N)(D)$ is the distribution of the population weighted for the distance it has moved towards or away from a given substance in that time (t). The details of this procedure have been presented (Castro et al., 1989).

Gradients. The salts KNO_3, NH_4NO_3, and NaOAc and the water-soluble substances glycerine and glycine all establish the same gradient under the same conditions of time and concentration. For example, the 22-hr, 10^{-2} M gradient for KNO_3 shown in Figure 1 is identical for all of these substances. Consequently, we assume the gradients for the other salts in this report are the same in this matrix. The concentration of radiolabeled substances in each 0.5-cm section of track was determined by liquid scintillation counting as previously described (Castro et al., 1989). Nitrate ion was determined spectrophotometrically by reduction to nitrite, diazotization of sulfanilamide, and coupling with *N*-(1-naphthyl)ethylenediamine (Chow and Johnstone, 1962). The intensity of

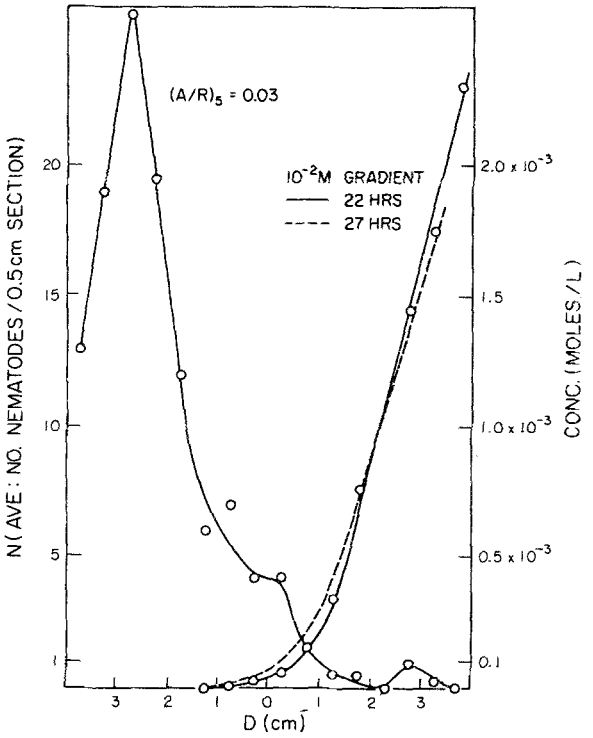

FIG. 1. Population distribution of MiJ_2 at 5 hr (left ordinate) in response to a 22-hr 10^{-2} M KNO_3 gradient (solid line, right ordinate) and the 27-hr gradient (dashed line, points not shown).

the band at 543 nm was determined with a Cary 118C spectrophotometer. In this work we have employed as standard an initial gradient of 10^{-2} M and a ΔT of 22 hr, that is, the end 0.5-cm section of track was made 10^{-2} M and the gradient was allowed to establish for 22 hr before inoculating the center of the track with larvae.

RESULTS AND DISCUSSION

Figure 1 shows the gradient (right ordinate) established with 10^{-2} M potassium nitrate at 22 hr (ΔT), and the population density of MiJ_2 5 hr after inoculation (t) (left ordinate). The gradient at 27 hr is also sketched in (dashed line). The N values plotted are an average of four replicate determinations. The concentration at the center that triggered this response was 4×10^{-5} M. It will be noted that the gradient does not change appreciably during the 5 hr allotted for

the population distribution of the nematode to develop. Clearly the larvae are strongly repelled by this salt.

The $(A/R)_5$ values for some representative salts of similar charge type are shown in Table 1. All initial gradients were 10^{-2} M. All salts containing K^+, NH_4^+, Cl^-, and NO_3^- are consistently repellent. The variation of $(A/R)_5$ with initial gradient concentrations of these salts is plotted in Figure 2. While the response to the individual salts is measurably different, we have chosen to sketch one line through all of the points to illustrate the general character of the response. Potassium nitrate is the most repellent and is represented by the lower points at each concentration. The concentration of the test substance at the center of each track, the triggering concentrations, at $\Delta T = 22$ hr for 5×10^{-3} M and 5×10^{-4} M gradients are 1×10^{-5} and 1×10^{-6} M respectively. For potassium nitrate, the triggering concentration of 10^{-6} M corresponds to 0.1 ppm. The results suggest these salts may be used to shield plant roots from nematode attack.

A clearer picture of the specificity of the response of these larvae to certain salts of the group IA alkali metals can be discerned from the data in Table 2. There are some trends in these responses. Cations showing little or no effect at these concentrations are Li^+, Na^+, and Rb^+. Repellent cations are K^+, CS^+, and NH_4^+. Employing the data of both Table 1 and Table 2, the following anions show little or no effect: F^-, HO^-, OAc^-, $Fe^{III}edta^-$, SO_4^{2-}, and PO_4^{3-}. Except for fluoride, the other halide ions are repellent, as is nitrate ion. A rough ordering of repellency from Table 2 for repellent anions would be: $NO_3^- > Cl^- > Br^-, I^-$. The repellent cations exhibit about the same potency, with potassium showing a slightly greater effect: $K^+ > CS^+, NH_4^+$. The chloride and nitrate salts of these ions are all strong repellents $(A/R)_5 < 0.1$.

TABLE 1. RESPONSE OF SECOND STAGE LARVAE OF *Meloidogyne incognita* TO VARIOUS SALTS: 10^{-2} M GRADIENTS, ΔT 22 hr

Salt	$(A/R)_5$
None	1.0 ± 0.2^a
NaOH	1.2
NaOAc	0.9
$NaFe^{III}edta$	1.1
$KFe^{III}edta$	0.5
NaCl	0.1
KNO_3	0.02
KCl	0.1
NH_4Cl	0.03
NH_4NO_3	0.06

[a] The ideal control response is 1.0. Thus, the first three salts exhibit no effect.

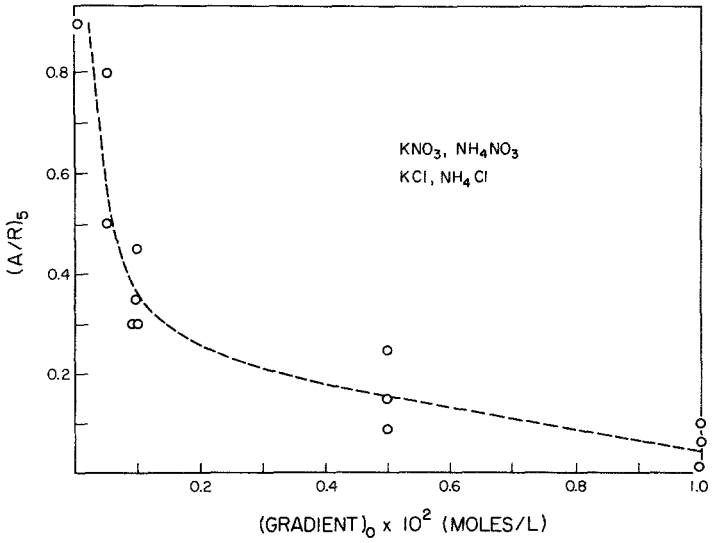

FIG. 2. Attractant/repellent ratios at 5 hr as a function of initial gradient concentration.

It is difficult to compare these results with previous related studies because the assay procedures employed were more qualitative in nature and they varied widely in design. Nevertheless, there are some interesting observations that should be noted. The bacteria-feeding nematode *Caenorhabditis elegans* was found to be attracted to a range of ions (Ward, 1973); these include Na^+, Li^+, K^+, Mg^{2+}, Cl^-, Br^-, I^-, and HO^-. Defined or calculated gradients were employed. The ions Na^+, Mg^{2+}, Cl^-, and OAc^- have also been reported to be attractive to the plant parasite *Rotylenchulus reniformis* (Riddle and Bird,

TABLE 2. $(A/R)_5$ VALUES FOR RESPONSE OF SECOND-STAGE LARVAE OF *Meloidogyne incognita* TO AMMONIUM AND OTHER ALKALI SALTS[a]

	Anions							
	F^-	Cl^-	Br^-	I^-	OAc^-	NO_3^-	SO_4^{-2}	PO_4^{-3}
Li^+	0.8	0.3	0.1	0.2	0.8	0.08		
Na^+	1.0	0.1	0.3	0.3	1.0	0.05	0.9	0.7
K^+	0.2	0.03	0.1	0.1	0.4	0.03		0.4
Rb^+	0.5	0.1	0.1	0.15_5	0.9	0.1		
CS^+	0.8	0.3	0.1	0.2	0.4	0.03		
NH_4^+	0.6	0.03	0.07	0.3	0.4	0.06	0.4	

[a] Reproducibility for $(A/R)_5 < 0.1$, $\pm 30\%$, for $(A/R)_5 > 0.1$, $\pm 20\%$.

1985). We find that none of these ions is attractive to *M. incognita*. Indeed K^+ and Cl^- are strongly repellent to this nematode. This opposite response to the same stimuli suggests a different set of molecular receptive sites are present in *M. incognita* or they reflect a different translation of the receptive event.

Differing responses of nematodes to metal ions has precedent in the work of Prot (1978a,b, 1979a,b). An examination of 12 "mineral salts" [NaCl, $NaNO_3$, NaH_2PO_4, KCl, KNO_3, KH_2PO_4, $CaCl_2$, $Ca(NO_3)_2$, $MgCl_2$, $MgSO_4$, $FeCl_2$, and $FeSO_4$] indicated all exhibited some degree of repellency toward *Meloidogyne javanica*, except perhaps $FeSO_4$. Moreover, Hoagland's solution [a solution containing all of the mineral salts noted above plus H_3BO_3, $MnSO_4$, $ZuSO_4$, $CuSO_4$ and $(NH_4)_6\ Mo_2O_{24}$] was reported to be repellent to larvae of both *M. javanica* and *M. incognita*. Finally, differences in the response of juvenile *Heterodera oryzae* and *Scutellonema cavenessi* to salts that repelled *M. javanica* were noted.

Our results are limited to *M. incognita*, but, in the main, we believe they are in qualitative agreement with the work of Prot. Some of the ions we have delineated as repellent were present in the salts or salt mixtures he employed. As we have shown here, all salts do not elicit a chemotactic response with this nematode.

An explanation of these effects awaits further study, but it is clear that no simple correlation with size or charge of the ions is possible. For example, the approximate ionic radii (Cotton and Wilkinson, 1988) increase in the series NH_4^+, $K^+ < Rb^+ < CS^+$. The response of infective nematodes to these and other salts is under investigation. Efforts to demonstrate the effectiveness of simple salts to shield plant roots from infection are also underway.

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CHEMICAL COMMUNICATION IN THE DACETINE ANT *Daceton armigerum* (HYMENOPTERA: FORMICIDAE)

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Abstract—Contrary to previous assumptions, *Daceton armigerum*, the largest ant in the myrmicine tribe Dacetini, employs trail communication. We identified two anatomical sources of trail pheromones: Trails drawn with poison gland contents can last for more than seven days. Trails drawn with the newly discovered sternal glands (in the VIth and VIIth abdominal sternites) are effective but relatively short-lived. In addition, our bioassays revealed that the contents of the mandibular glands elicit alarm behavior, and secretions from the pygidial gland release attraction. When tested with artificial poison gland trails from seven other myrmicine species, *Daceton* did not exhibit trail following behavior. We confirmed, however, previous findings that *Atta* respond to *Daceton* poison gland trails and *Solenopsis* follow *Daceton* Dufour's gland trails.

Key Words—Ants, Dacetini, *Daceton armigerum*, Hymenoptera, Formicidae, poison gland, pygidial gland, sternal gland, mandibular gland, trail communication, alarm communication.

INTRODUCTION

The myrmicine tribe Dacetini is worldwide in distribution, and its 24 genera and 250 species vary enormously in size, morphology, and behavior. The analysis by Brown and Wilson (1959) of the Dacetini was one of the first attempts to correlate the evolution of social behavior with species-level adaptations in feeding and has been supplemented by more recent studies by Dejean (1980a,b; 1985a,b) and Masuko (1984) (see review in Hölldobler and Wilson, 1990).

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These comparative studies suggest an evolutionary tendency of dacetine species to move from open, even partly arboreal foraging, to cryptic mostly terrestrial and subterranean foraging. The ancestral habits may be retained in the genera *Daceton* and *Orectognathus* today. In fact, *Daceton armigerum*, the only species of the genus, is strictly arboreal and has been considered the most primitive dacetine species (Brown and Wilson, 1959; Wilson, 1962). A more detailed study of these genera is therefore of special interest.

The division of labor within colonies of *Daceton* and *Orectognathus versicolor* has been studied by Wilson (1962) and Carlin (1981), respectively. In addition, Hölldobler (1981) discovered in *O. versicolor* chemical communication employed during nest emigrations and alarm behavior. These genera have the most complex division of labor known for the Dacetini. Very little, however, has hitherto been reported on the communication behavior in *Daceton armigerum*.

In the present laboratory study of *D. armigerum* we investigated the glandular morphology of some of the major cuticular glands and conducted a series of bioassays testing the possible behavioral functions of some of the exocrine glandular secretions. The results reveal a new trail pheromone gland in ants and a remarkably diverse array of chemical communication signals.

METHODS AND MATERIALS

A colony of *Daceton armigerum* was collected on May 6, 1988, at the Imataca Forest Reserve, east of El Palmar at the border between Bolivar State and Delta Amacuro Territory, Venezuela. The complete colony contained one dealate queen and 2342 adult workers nesting 6.5 m above ground within a hollow in a tree trunk. Workers outside the nest moved primarily on a route at least 22 m in length, which was stable over the two days of observations. A large subsample of the colony was returned to Cambridge for study.

Worker subcastes were distinguished by size: minors (head width less than 2.2 mm), medias (head width 2.2–4.2 mm), and majors (head width more than 4.2 mm). In the laboratory at Harvard University, the colony was housed in 30 test tubes that had water trapped behind cotton plugs to provide moisture. The nest tubes were piled into two plastic containers (37 × 26 cm). From there the ants had access through cardboard bridges to a large foraging area (150 × 75 cm), where they were offered freshly killed cockroaches, frozen crickets, honey water, and a specially prepared diet (Bhatkar and Whitcomb, 1970). Unfortunately, the queen died several days after the colony had been transported to the laboratory. Although the whole group slowly declined in numbers to about 200 workers when we terminated the experiments, the loss of the queen did not result in a major social disintegration. The workers continued actively patrolling

and foraging in the arena. Some nest workers became fertile, laying viable eggs, which developed into males. Dissections revealed that the workers have two to four ovarioles, each of which can contain one large oocyte.

For histological investigations specimens were preserved in Carnoy's fixative and stored in 80% ethanol. After clearing in toluene, the gasters of workers were embedded in an ultra-low-viscosity epoxy medium (VCD/HXSA) as described by Mascorro et al. (1976) and Oliveira et al. (1983). Blocks were serially sectioned at 2 μm using glass knives and a model MT2-C ultramicrotome (Research Manufacturing Company, Tucson, Arizona). Sections were attached to albuminized glass slides, and plastic was removed prior to staining with toluidine blue–basic fuchsin following Burns and Bretschneider (1981) with slight modifications. Scanning electron micrographs were taken with an AMR 1000A SEM.

Descriptions of individual bioassays are given in the appropriate sections below.

RESULTS

The *Daceton* workers were active in the foraging arena during the daytime with peak activity in the afternoon, and they returned to the nest tubes during the night. Among the worker subcastes, we primarily saw the majors and several size classes of the media groups patrolling the arena and foraging. The minors were rarely seen outside the nest tubes. We did not observe a noticeable recruitment effect when we offered as food honey water (which was readily imbibed by foragers and exchanged among nest mates by regurgitation), or cockroaches and crickets. However, on three occasions (of a total of nine incidents) when we presented termites as prey objects, we noticed that after 10–20 min the number of ants moving over the cardboard bridge into the arena increased markedly. In this situation most ants followed a reasonably well defined route from the nest to the food source in the arena. In fact we observed several workers apparently laying down pheromones. They moved more slowly with their bodies lowered to the ground, so that the last abdominal sternites could touch the surface (Figure 1a). With the aid of a movable operation microscope (Technoscope Zeiss), we were able to confirm these observations and, in addition, noticed that some of the workers extended their sting at irregular intervals.

The observations strongly suggested that *Daceton armigerum* employ chemical recruitment signals and exhibit trail following behavior. The following investigations were designed to test this hypothesis.

Gland Morphology. Daceton workers have a large poison gland that spans approximately one third the length of the gaster. The relatively wide tube lead-

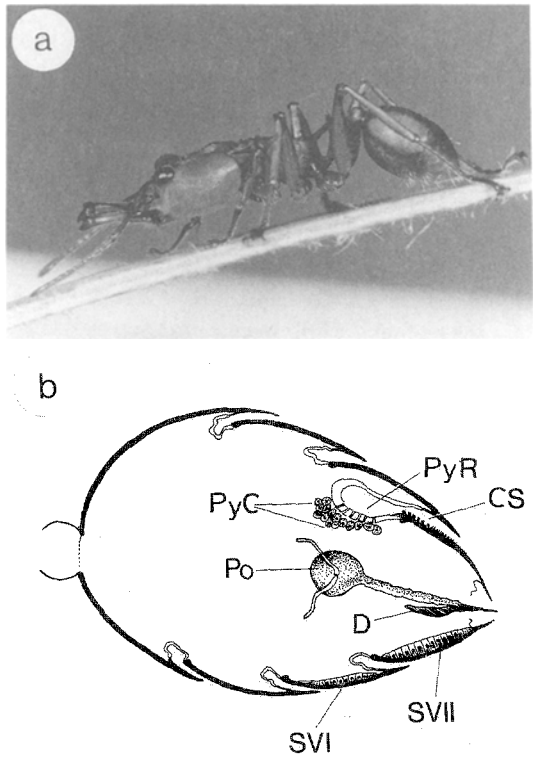

FIG. 1. (a) A worker of *Daceton armigerum* moving over the bridge connecting the nest and foraging arena. The ant closely inspects the substrate with its antennae, apparently following a trail. Its body is lowered, so that the last abdominal sternite can touch the surface. (b) Schematical illustration of a sagittal section through the gaster of a *D. armigerum* worker showing the major exocrine glands. **PyR,** pygidial gland reservoir; **PyC,** pygidial gland cells; **Po**, poison gland; **D,** Dufour's gland; **SVI,** sternal gland in VIth abdominal sternite; **SVII,** sternal gland in the VIIth abdominal segment; **CS,** cuticular structure.

ing from the reservoir to the sting is filled with secretions and appears to be bulging along its entire length. The Dufour's gland is slender and approximately one third the length of the poison gland (Figure 1b).

Histological and scanning electron microscopic investigations revealed a large, paired pygidial gland. The gland reservoir sacs are formed as invaginations of the intersegmental membrane between the VIth and VIIth abdominal tergites (Figure 2). Ducts lead from clusters of glandular cells that penetrate the intersegmental membrane into the reservoir (Figure 4a). The cuticle of the adjacent VIIth tergite has a complex surface structure, which also occurs in a paired arrangement (Figures 3 and 4). Presumably the secretions of the pygidial gland

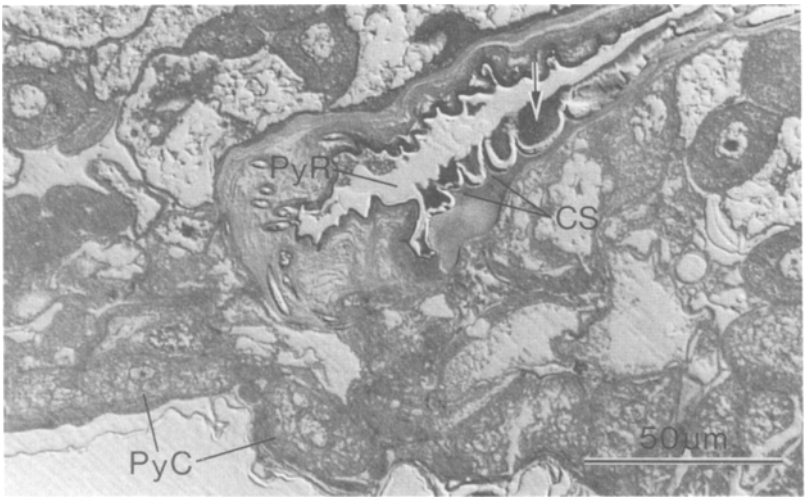

FIG. 2. Sagittal section through the pygidial gland of a *Daceton armigerum* worker. **PyR,** pygidial gland reservoir; **PyC,** pygidial gland cells; **CS,** cuticular structure on the VIIth abdominal tergite; arrow indicates coagulated secretions in the grooves of the cuticular structure.

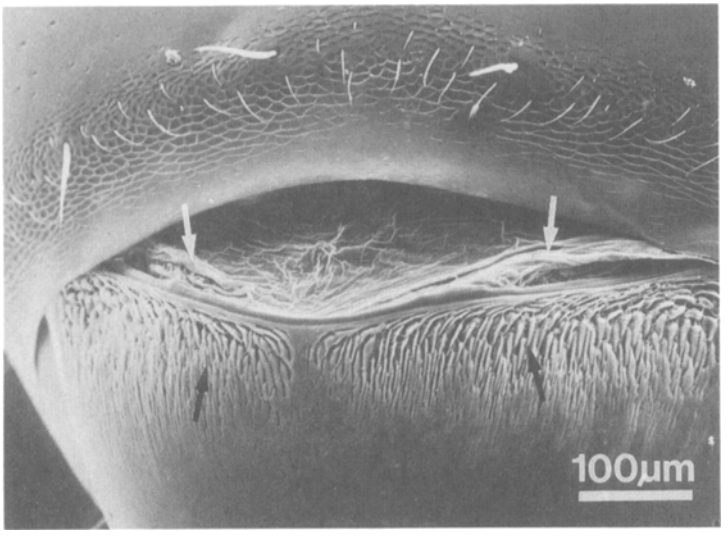

FIG. 3. Scanning electron micrograph of the pygidial gland opening showing the paired cuticular structure (black arrows) on the VIIth abdominal tergite and the intersegmental membrane (white arrows) between the VIth and VIIth abdominal tergites.

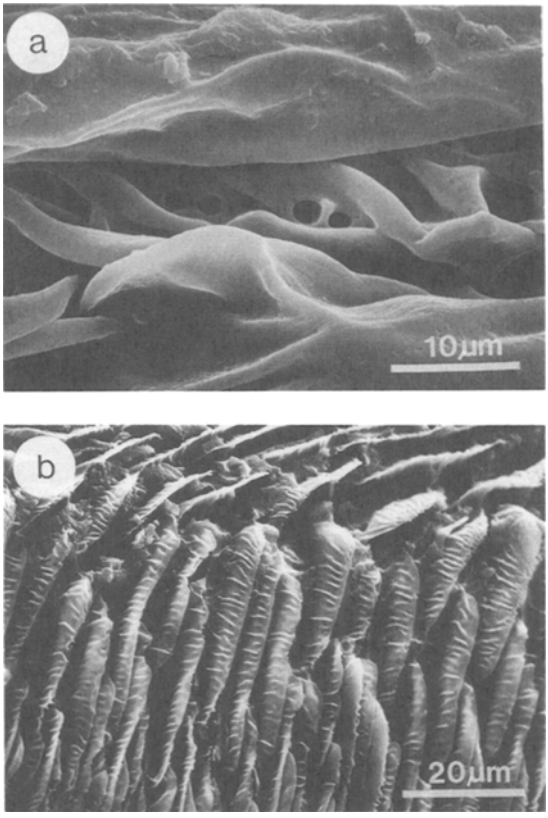

FIG. 4. Higher magnification scanning electron micrographs of details designated by arrows in Figure 3. (a) Area of intersegmental membrane (between abdominal tergites VI and VII) showing pygidial gland duct openings. (b) Grooves of the cuticular structure on abdominal tergite VII.

reservoir can be released into the grooves of this cuticular structure, which, because of its convoluted design, might direct and facilitate the dispersion of the glandular product. In fact, in the histological preparations, one can see coagulated secretions inside the cavities of the cuticular structure (see Figure 2).

Daceton workers possess large glandular epithelia in the last two exposed abdominal sternites. The most posterior sternal gland is especially well developed (VIIth sternite). The epithelium is attached to the cuticle. Intracellular ducts connect to very fine pore capillaries that penetrate the cuticle in dense formation (Figure 5).

Bioassays of Glandular Secretions: Trail Following. In a series of exper-

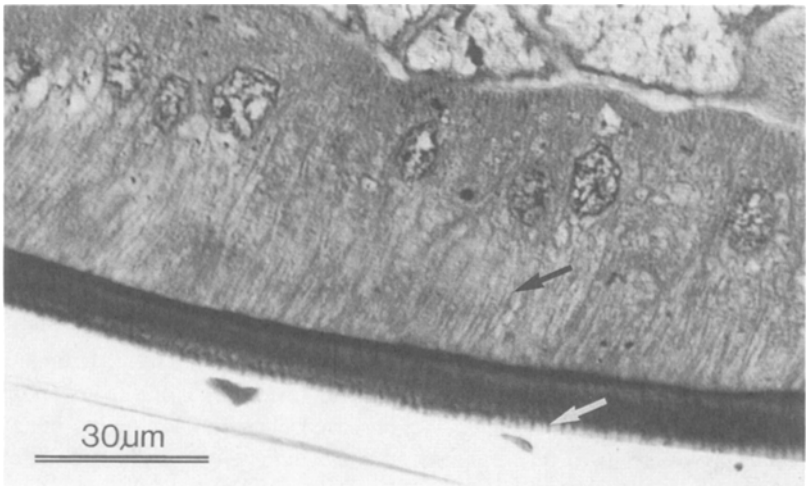

FIG. 5. Sagittal section of the glandular epithelium in the VIIth abdominal sternite (sternal gland). Black arrow points to an intracellular channel. White arrow indicates the opening of a pore capillary in the cuticle.

iments we tested the trail-following response of *Daceton* workers to artificial trails drawn with glandular secretions of poison gland, Dufour's gland, pygidial gland, sternal gland, and hind gut contents. The organs were dissected out of ants killed by placing them for a few minutes in a freezer. For each trail test, one gland of a kind was crushed on the tip of a hardwood applicator stick and smeared once along a 20-cm-long pencil line. The trails were made to originate either from the entrance of a nest tube or from the base of the bridge that connected the nest box with the foraging area. As a control, one or several trails were offered simultaneously that derived either from a droplet of water or from one of the other glands. All ants following the trails to the end during the first 5-min period were counted.

As can be seen from Table 1, trails drawn with crushed poison glands or sternal glands (VIIth abdominal sternite) elicited a precise trail-following behavior in *Daceton* workers (Figure 6), but the ants did not follow trails drawn with crushed Dufour's gland, pygidial gland, or hindgut contents.

All worker subcastes have the same glandular equipment, and their secretions release the same behavioral responses. Although there was unequivocal response to trails drawn with sternal glands, the response to poison gland trails was usually stronger ($P < 0.01$; t test; Table 1). Furthermore, trails drawn with crushed sternal glands were only effective for several minutes, whereas poison gland trails can last for several days. In a series of experiments, we

TABLE 1. MEAN NUMBER AND STANDARD DEVIATION ($N = 9$) OF *Daceton armigerum* WORKERS FOLLOWING ARTIFICIAL TRAILS DRAWN WITH CRUSHED EXOCRINE ORGANS DURING 5-MIN PERIOD

Poison gland	Dufour's gland	Pygidial gland	Hindgut	Sternal gland (VIIth sternite)
17.3 ± 9.4	0	0	0	6.3 ± 3.8

tested the trail-following response along poison gland trails of different age. Each trail was drawn with one crushed gland and kept in room temperature (22°C) for increasingly extended intervals (ranging from 1 min to seven days) until it was presented to the ants. We observed clear trail-following behavior along 7-day-old trails (5.0 ± 2.9 ants; $N = 5$). In fact, it is likely that the trails

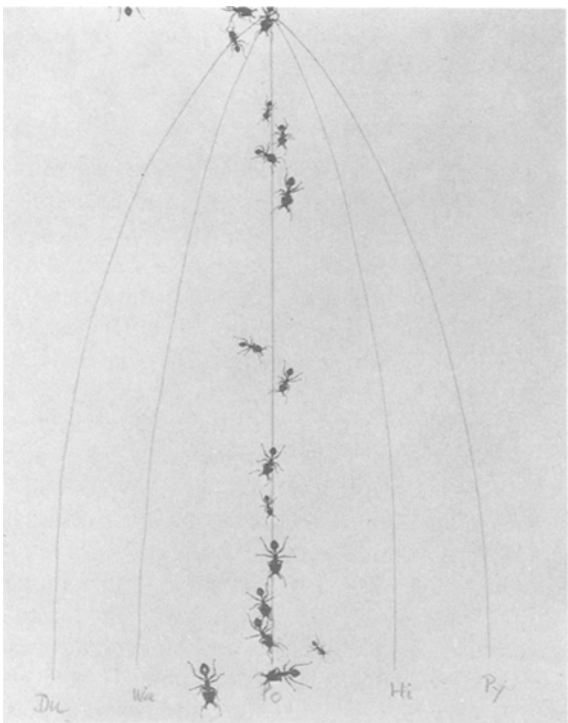

FIG. 6. Trail following response of *Daceton armigerum* workers when five artificial trails were offered at the base of the arena bridge. **Du,** crushed Dufour's gland; **Wa,** water; **Po,** crushed poison gland, **Hi,** crushed hindgut; **Py,** crushed pygidial gland.

TABLE 2. TRAIL-FOLLOWING RESPONSE OF *Atta cephalotes* WORKERS ALONG TRAILS DRAWN WITH GLANDULAR SECRETIONS FROM *Daceton armigerum* AND *A. cephalotes*

Daceton poison gland	vs. *Daceton* Dufour's gland
30.3 ± 17.3[a]	0
	vs. *Atta* poison gland
13.6 ± 4.3[b]	39.2 ± 7.4

[a] $N = 6$.
[b] $P < 0.002$ (paired t test); $N = 5$.

last even longer, because in one test the ants followed a trail which was drawn 10 days previously.

From these experiments we conclude that *Daceton* produce trail pheromones in the poison gland and in the sternal gland. These results differ from previous findings by Blum and Portocarrero (1966), who failed to demonstrate trail-following in *D. armigerum*. Interestingly, however, these authors found that three attine ant genera follow trails drawn with poison gland secretions of *D. armigerum*. Similarly, Wilson (1962) discovered that *Solenopsis invicta* followed trails drawn with Dufour's gland contents of *D. armigerum*.

Our studies confirmed these findings of interspecific responses. We offered *Atta cephalotes* and *Solenopsis invicta* trails drawn with either poison or Dufour's gland secretions of *D. armigerum*. As Tables 2 and 3 show, *Atta* follow poison gland trails and ignore Dufour's gland trails, whereas *Solenopsis* follow the Dufour's gland trails but not the poison gland trails. However, when we offered *Solenopsis* or *Atta* a choice between the effective *Daceton* trail and a trail drawn with the test species' own trail gland, the ants significantly preferred their own species' trails. *Daceton*, on the other hand, is very specific in its trail-following response. It only followed its own poison gland secretions,

TABLE 3. TRAIL-FOLLOWING RESPONSE OF *Solenopsis invicta* WORKERS ALONG TRAILS DRAWN WITH GLANDULAR SECRETIONS FROM *Daceton armigerum* AND *S. invicta*

Daceton poison gland	vs. *Daceton* Dufour's gland
0[a]	32.8 ± 16.4
Daceton Dufour's gland	vs. *Solenopsis* Dufour's gland
15.9 ± 6.3[b]	42.2 ± 9.2

[a] $N = 5$
[b] $P < 0.001$ (paired t test); $N = 9$.

ignoring those of *Atta cephalotes*, *Solenopsis invicta*, *Tetramorium caespitum*, *Aphaenogaster cockerelli*, *Pheidole desertorum*, *Pheidole dentata*, *Pogonomyrmex occidentalis*, and *Pogonomyrmex badius*.

Bioassay of Glandular Secretion: Chemical Alarm and Attraction. Although *Daceton* did not follow trails drawn with crushed pygidial glands, we noticed an attraction to pygidial gland secretions. This was revealed by the following bioassays:

We offered at the entrance of a nest tube alternately either an applicator on which we had crushed the Vth abdominal tergite (control) or an applicator on which we had crushed the VIIth tergite (with the pygidial gland attached). The applicators were held approximately 1 cm above the surface so that the ants could not touch them. The number of ants were counted that left the nest tube within a 2-min period after the applicators were presented.

From a total of six tests, the mean number of ants leaving the nest tube when the control was offered was 4.8 ± 5.5 ($\bar{X} \pm$ SD), which was significantly ($P < 0.02$; paired t test) different from the mean number of leaving ants (13.3 ± 5.9) when crushed pygidial glands were offered. The response toward pygidial gland secretions not only was attraction, but also an increase in locomotory speed.

We also noticed that the mandibular gland contents had a strong smell. When filter paper on which mandibular glands or whole heads of *Daceton* workers were crushed were presented to the ants, they responded with attraction, increase in locomotory speed, and aggression, charging the paper with widely opened mandibles. In another experimental series, two applicator sticks, one plain, the other contaminated with the mandibular gland secretions of one ant, were placed into the arena (approximately 10 cm apart). After 60 sec the number of ants assembled at the sticks were counted. From a total of 10 tests, the mean number of ants assembled at the contaminated stick was 10.6 ± 3.7 ($\bar{X} \pm$ SD). This was significantly ($P < 0.001$; paired t test) different from the mean number of ants at the control stick (2.4 ± 2.3). At the contaminated stick the ants behaved aggressively, charging and biting the stick repeatedly.

Finally, we tested the response of *Daceton* workers towards Dufour's gland secretions. As already reported, *Daceton* workers did not follow trails drawn with Dufour's gland secretions. Neither did they exhibit an obvious alarm behavior when exposed to crushed Dufour's glands. However, when the secretion was offered on small disks (4 cm diameter) of filter paper in the arena (eight repetitions), the mean number of ants inspecting these disks with their antennae in five consecutive snapshot counts during a 5-min period was significantly higher (3.3 ± 0.5) than the mean number of ants inspecting a simultaneously offered control paper (0.8 ± 0.3; $P < 0.001$; paired t test). Thus, *Daceton* workers pay some attention to the Dufour's gland secretions, but it is not clear to us whether this secretion has signal function and in what context it might be discharged.

DISCUSSION

Daceton armigerum are the biggest ants in the myrmicine tribe Dacetini, living in large arboreal colonies. Almost nothing was known about communication in this species. We were able to study the chemical communication in the laboratory using a large portion of a *D. armigerum* colony.

Although it has been well documented that many species of the subfamily Myrmicinae employ secretions from the glands associated with the sting apparatus (poison gland; Dufour's gland) for chemical trail communication and orientation (for review see Morgan, 1984; Attygalle and Morgan, 1985; Hölldobler and Wilson, 1990), our finding of trail communication in *Daceton* is only the second case in the tribe Dacetini where this behavior has been demonstrated. In fact, previous studies have failed to show trail following in *D. armigerum*, and therefore it was assumed that this species has no trail communication (Blum and Portocarrero, 1966). A surprising result was the astounding persistence of the poison gland trails of *Daceton*. Trails older than one week still elicited a precise trail-following behavior in *D. armigerum* workers.

Although we did not find *Daceton* workers following trails drawn with Dufour's gland secretions, the Dufour's gland contents of *D. armigerum* are followed by *Solenopsis invicta* (Wilson, 1962), while the poison gland contents are followed by species of *Acromyrmex* and *Atta* (Blum and Portocarrero, 1966). We were able to confirm these interspecific responses. These results suggest that the Dufour's gland of *D. armigerum* contains the farnesene known as a major component of the *Solenopsis* trail pheromone (Vander Meer, 1986) and its poison gland the pyrrole, one of the active trail pheromone components identified in attine ants (Tumlinson et al., 1971, 1972). On the other hand, the trail-following response of *D. armigerum* is highly specific: they do not follow poison gland secretions of *Atta* or of seven other myrmicine species tested.

We discovered large glandular epithelia in the VIth and VIIth abdominal sternites. This structure is especially well developed in the VIIth sternite, where one can detect intracellular ducts connecting with fine pore capillaries in the cuticle. Sternal epithelial glands are known from several ant species (Hölldobler and Engel, 1978; Jessen et al., 1979), but this is the first case where a behavioral response to its secretions could be demonstrated: Workers follow trails drawn with secretions from the VIIth sternite glands. We assume that both poison gland and sternal gland secretions might be involved in trail formation, because behavioral observations indicate that trail-laying ants touch the last abdominal sternites to the ground and at irregular intervals extrude the sting. In fact, in our tests, when we offered poison gland and sternal gland secretions on the same trail, we observed the most precise trail-following behavior, although the number of ants following the trail was not significantly different from those following poison gland trails alone.

There is also the possibility that a recruitment signal is discharged from

the well developed pygidial gland. The secretion of this gland elicits attraction, but no trail-following behavior. In the dacetine species *Orectognathus versicolor* Hölldobler (1981) found workers following pygidial gland trails. In this species the pygidial gland is associated with an epithelial tergal gland in the VIIth tergite (which is absent in *Daceton*). It is possible that in this case the epithelial tergal gland has the same function as the epithelial sternal gland in *Daceton* (which is absent in *Orectognathus*).

Based on the findings in *O. versicolor*, where we discovered the pygidial gland associated with a glandular epithelium in the VIIth abdominal tergite, which resembles pygidial glands in some ponerine ants (i.e., *Pachycondyla laevigata*; Hölldobler and Engel, 1978), Hölldobler (1981), we speculated that the closely related *Daceton* might also have a well-developed pygidial gland of similar construction. In the present paper, we confirm that *Daceton* possesses a well-developed pygidial gland, which resembles that of *Orectognathus*; however, the glandular epithelium in the VIIth abdominal tergite is absent.

In the first behavioral study of *Daceton*, Wilson (1962) observed that workers of *D. armigerum* often moved to areas of excitement, and when a worker ant had discovered prey it moved in "excited broken running patterns" by which other ants in the vicinity might be attracted. Wilson (1962, 1971) hypothesized that this running pattern might serve as a communicative signal of the kind of "Stäger's kinopsis," i.e., the large-eyed *Daceton* worker might respond to the visual stimuli produced by the nestmate moving excitedly. There is no doubt *Daceton* has excellent vision and readily reacts to moving objects in its visual field. Our new results on the response to chemical signals from the mandibular glands and several abdominal glands suggest that these glands are also involved in this behavior. Indeed, chemical communication in *Daceton* is well developed and plays a major social role in this species.

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INACTIVATION OF BACULOVIRUS BY QUINONES FORMED IN INSECT-DAMAGED PLANT TISSUES

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Abstract—The infectivity of the nuclear polyhedrosis virus, HzSNPV to *Heliothis zea* was significantly reduced when viral occlusion bodies were exposed to the plant phenolic chlorogenic acid in the presence of polyphenol oxidase. Chlorogenic acid is rapidly oxidized to the ortho-quinone, chlorogenoquinone, by foliar polyphenol oxidases of the tomato plant, *Lycopersicon esculentum*, when foliage is damaged during feeding by larval *H. zea*.

Our results indicate that chlorogenoquinone, a powerful oxidizing agent, covalently binds to the occlusion bodies of HzSNPV and significantly reduces their digestibility and solubility under alkaline conditions. This binding is proposed to interfere with the infection process by impairing the release of infective virions in the midgut.

Key Words—Baculovirus, nuclear polyhedrosis virus, plant phenolics, polyphenol oxidase, tritrophic interactions, *Heliothis zea*, Lepidoptera, Noctuidae, *Lycopersicon esculentum*, host plant resistance, biological control.

INTRODUCTION

A compelling body of evidence implicates the host plant of insect herbivores as a significant determinant in their susceptibility to disease (Schultz, 1983; Jones, 1983; Keating and Yendol, 1987; Keating et al., 1988; Richter et al., 1987; Ramoska and Todd, 1985; Hare and Andreadis, 1983; Noguchi and Yamaguchi, 1984; Faeth and Bultman, 1986; Berenbaum, 1988; Barbosa, 1988; Shepard and Dahlman, 1988). An array of phytochemicals, including phenolics, alkaloids, nonprotein amino acids, and terpenoids may influence the susceptibility of insects to bacterial diseases (Koike et al., 1979; Salama et al.,

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1984; Felton and Dahlman, 1984; Krischick et al., 1988), viral diseases (Felton et al., 1987; Keating et al., 1988), microsporidia (Smirnoff, 1967), and fungal diseases (Gochnauer et al., 1979).

Differences in susceptibility to a given pathogen have most often been attributed to dietary stress (Noguchi and Yamaguchi, 1984; Felton and Dahlman, 1984; Stubblebine and Langenheim, 1977) or direct antimicrobial activity of plant factors (Gochnauer et al., 1979; Felton et al., 1987; Keating et al., 1988). Knowledge of the specific mechanisms by which stress or phytochemicals affect the incidence of disease in insects is meager. One of the most explicit examples of the influence of phytochemicals concerns *Bombyx mori*, showing the antibacterial action of caffeic acid against *Streptoccus faecalis* (Koike et al., 1979; Iizuka et al., 1974) and the antiviral action of a chlorophyll-protein complex formed in the larval gut (Hayashiya, 1978).

In previous studies, the predominant orthodihydroxyphenols, rutin and chlorogenic acid, from the tomato plant, *Lycopersicon esculentum*, inhibited the infectivity of NPV in larval *Heliothis zea* and in cell culture of *Trichoplusia ni* (Felton et al., 1987). Here, we extend our analysis of the inhibitory influence of phenolics on NPV to account for the effect of plant oxidative enzymes. Besides high constitutive levels of rutin and chlorogenic acid, tomato foliage contains high levels of polyphenol oxidase (PPO) and peroxidase (POD) activities (Felton et al., 1989; Duffey, 1986; Duffey and Felton, 1989). When foliage is physically damaged, such as by chewing of insect larvae, these oxidases rapidly convert chlorogenic acid to the *o*-quinone, chlorogenoquinone (Felton et al., 1989). Moreover, this oxidation continues during the digestion of the food in the midgut (Felton et al., 1989). The *o*-quinones are noted for their ability to conjugate rapidly with nucleophilic $-NH_2$ and $-SH$ groups of amino acids, peptides, and proteins (Hurrell et al., 1982; Cheynier et al., 1988; Pierpoint, 1966, 1969). This conjugation has been linked to the ability of plants to resist viral diseases (Pierpoint et al., 1977), fungal diseases (Gentile et al., 1988; Ampomah and Friend, 1988), and nematode infections (Brueske and Dropkin, 1973).

We show here that the binary action of phenolics and polyphenol oxidase significantly reduces the infectivity of NPV in larval *H. zea*. The significance of these findings to ecological theory and host-plant resistance is discussed.

METHODS AND MATERIALS

Insects and Virus. Eggs of *Heliothis zea* were obtained from the USDA Southern Field Crop Management Laboratory, Stoneville, Mississippi. Larvae were maintained in an environmental chamber at 27°C with a 16:8 light-dark

photoperiod in 25-well plastic rearing trays obtained from Bio-Serv Inc. (Frenchtown, New Jersey). Larvae were fed semidefined artificial diet (No. 976L3) purchased from Bio-Serv Inc. with streptomycin sulfate (100 ppm) and 5.5% aureomycin (1000 ppm) added as antibiotics.

Occlusion bodies of *Heliothis zea* nuclear polyhedrosis virus (HzSNPV) were obtained from Sandoz-Wander, Co., washed in ddH_2O, pelleted at 10,000*g*, and lyophilized.

Chemicals. Chlorogenic acid (CHA) and PPO (mushroom tyrosinase) were purchased from Sigma Chemical Co., St. Louis, Missouri. [^{3}H]Chlorogenic acid was purchased from Research Products International Corp. (91191 Gif-sur-Yvette, France) and was purified following Isman and Duffey (1983) to a specific activity of 0.7 mCi/mmol. Radioactivity was determined using a Beckman LS 230 liquid scintillation counter. An aqueous scintillant was used (ACS, No. 196290, Amersham Corp. Arlington Heights, Illinois), and total radioactivity was determined using the channel-ratios method.

Measurement of Polyphenol Oxidase Activity. Tomato foliage was homogenized in 0.1 M potassium phosphate buffer, pH 8.0, containing 7% polyvinylpolypyrolidine at 0–4°C. The homogenate was centrifuged at 11,000*g* for 10 min, and the supernatant was used immediately as the enzyme source. Polyphenol oxidase activity (as CHA oxidase) was measured spectrophotometrically (Ryan et al., 1982) by recording the increase in OD_{470}. One unit of PPO was defined as enzymatic activity producing change in OD_{470}/min = 0.001 under the assay conditions described by Ryan et al. (1982). Protein concentration was measured after Bradford (1976) with bovine serum albumin as a standard.

Amino Acid Analysis. To determine the effect of oxidation of CHA on the amino acid composition of occlusion bodies (OBs), 300 mg of purified OB in 50 mM Na_2CO_3 buffer, pH 8.1, 0.25 mM NaCl with 30 mg chlorogenic acid, and 750 units PPO (tyrosinase) were mechanically stirred for 2 hr. The OBs were pelleted at 10,000*g*, lyophilized, and dialyzed against ddH_2O. Amino acid analyses were performed with a Durrum D-500 amino acid analyzer (Sunnyvale, California). The treated OBs were compared to control OBs treated with heat-inactivated tyrosinase and chlorogenic acid.

Dissolution Kinetics. To determine the effect of oxidation of chlorogenic acid on the solubility of OBs, 20 mg of OB's pretreated with chlorogenic acid and/or tyrosinase in 0.01 M Tris buffer, pH 7.0 for 1 hr, at concentrations described in the above experiments, were solubilized in 0.5 ml DAS buffer (50 mM Na_2CO_3, 50 mM NaCl, 2 mM Na_4-EDTA, pH 10.6) after Whitt and Manning (1987). The buffer solubilizes polyhedral protein without affecting other viral proteins or nucleic acid. The dissolution of OBs was stopped at intervals of 0, 5, 10, 15, and 30 min with 2.5 ml of 0.1 M Tris HCl, pH 7.0. The preparations were then vortexed for 10 sec and centrifuged 10 min at 12,000*g*.

The protein concentration of the supernatant obtained at each timed interval was determined after Bradford (1976). Assays were replicated three times.

Digestibility of Occlusion Bodies. The digestibility of occlusion bodies treated with CHA and PPO as described above was determined using trinitrobenzenesulfonic acid (TNBS) to measure liberated amines (Fields, 1972). Insect protease was obtained from larvae following Broadway and Duffey (1986). Twenty milligrams of OBs were treated with one midgut equivalent in 10 ml 0.1 M phosphate buffer, pH 8.0, for 2 hr. Digestion was stopped with the addition of 1 ml 20% trichloroacetic acid. Samples were centrifuged at 20,000*g* for 30 min and free amines in the supernatant were measured by TNBS. Treatments were replicated five times.

Estimation of Binding of Chlorogenoquinone to HzSNPV. To determine if chlorogenic acid binds covalently to the occlusion bodies of HzSNPV, viral OBs were incubated for 1 hr in the presence of 100 units PPO, three levels of CHA, and 0.1 μCi [^{3}H]CHA in 40 ml 0.20 M phosphate buffer, pH 7.0. CHA concentrations tested were 1.41, 5.65, and 11.29 μmols/ml, representing a range of concentrations normally found in foliage (unpublished data). Following incubation, OBs were pelleted by centrifugation at 10,000*g* for 15 min, washed with 40 ml 8 M urea to remove noncovalently bound CHA (Barbeau and Kinsella, 1983) and centrifuged. This process of washing in urea was repeated at least five times until radioactivity of the supernatant equalled background levels. Finally, the OBs were washed in ddH_2O, pelleted by centrifugation a total of three times, and then frozen, lyophilized, and weighed. The OBs were hydrolyzed in 1 ml conc. HCl at 60°C for 2 hr. The OBs were then neutralized with NaOH, and color was bleached with the addition of a small amount of 30% H_2O_2. Hydrolysate was added to ACS counting fluid, counted in the scintillation counter, and the dpm calculated by the external standards method.

To determine the ability of CHA, in the presence of variable levels of PPO activity, to bind to polyhedrin, a modification of an equilibrium dialysis procedure (Barbeau and Kinsella, 1983) was performed. First, polyhedrin was isolated by incubation of OBs in DAS buffer for 24 hr at 4°C. Insoluble viral particles were pelleted by centrifugation at 20,000*g* for 30 min. Supernatant was used as source of polyhedrin. Protein concentration was estimated after Bradford (1976) with bovine serum albumin as a standard. Binding studies were performed with acrylic dialysis cells and membranes with a 10,000- to 12,000-mol wt cutoff. In each cell, 4 ml of 3.5 mM CHA in 0.1 M phosphate buffer, pH 7.0, and 0.05 μCi [^{3}H] CHA was added to one compartment. To the other compartment, separated from the first by dialysis membrane, 10 mg polyhedrin in 4 ml buffer was added with 0, 11.7, 23.5, 47, and 94 units PPO/ml buffer. Control cells were identical except that polyhedrin was absent. Dialysis cells were incubated at room temperature with mechanical shaking for 24 hr to reach

equilibrium. Duplicate 0.5-ml aliquots were then removed from each compartment of a cell, and radioactivity was directly counted in aqueous scintillation fluid. Estimation of binding was calculated by subtracting the differences in DPM between compartments and between experimental and control cells:

$$[\mathrm{dpm}_{\mathrm{PPO+POLYHEDRIN}} - \mathrm{dpm}_{\mathrm{CHA}}] - [\mathrm{dpm}_{\mathrm{PPO}} - \mathrm{dpm}_{\mathrm{CHA}}]$$

This method measures total binding (i.e., covalent and noncovalent interactions) because noncovalent interactions were not excluded by treatment with urea as in the earlier experiment.

Effect of Oxidation of Chlorogenic Acid on Viral Infectivity. To determine if the oxidation of CHA affects the infectivity of NPV, 0.0625 mg OB, 11,200 units PPO (tyrosinase), and 1.0 g casein in 100 ml ddH_2O, pH 7.0, were incubated with 0, 125, 250, 500, 1000, or 2000 mg CHA. Solutions were stirred for 1 hr, frozen, and lyophilized. The treated protein and OBs were added to artificial diet to give a concentration of virus of 500 OB/g diet at 0, 0.7, 1.4, 2.83, 5.66, and 11.32 μmol CHA/g diet. These levels of CHA and PPO represent the normal range found in field-grown plants (unpublished data).

Newly molted third-instar *H. zea*, obtained from the colony reared on untreated artificial diet, were placed on the respective diet for 48 hr. Previous results have shown that the presence of CHA and PPO in artificial diet does not retard larval feeding and hence viral acquisition. Larvae were then returned to untreated artificial diet for the duration of the experiment. Mortality was monitored daily until pupation. A total of 20–25 larvae per concentration of CHA were tested with three replicates.

To determine whether the oxidation of CHA in tomato foliage affects NPV infectivity, greenhouse-grown *L. esculentum* var. Ace 55 was used in bioassays. Leaflets were excised from greenhouse plants at the six- to 8-leaf stage and transported to the laboratory on ice. Foliage was divided into two treatments. In the first treatment, PPO was not inhibited by the addition of inhibitors until substantial oxidation had occurred. In this treatment, 100 g (wet weight) of foliage was homogenized in a blender for 1 min in 200 ml of ice-cold ddH_2O, adjusted to pH 7.0 with 6 N NaOH, transferred to a 500-ml covered beaker, and mechanically stirred for 2 hr at room temperature with 135 OB/g foliage. The 2-hr stirring period was chosen because it represents the minimal time that larvae retain tomato foliage in their digestive system (Felton et al., 1989). However, more than 90% of tomato PPO activity normally occurs during the initial 15 min following maceration. After stirring for 2 hr to allow oxidation, the PPO inhibitor phenylthiourea (50 mg) and a reducing agent, ascorbic acid (150 mg), were added, and the solution was stirred for an additional 10 min. The solution containing the foliage and PPO inhibitors was frozen and lyophilized. Dried foliage was reconstituted in H_2O and 2.5% agar to the original weight of 100

g and fed to newly molted third-instar larvae for 48 hr. Larvae were then returned to artificial diet as described.

In the second treatment, oxidation was inhibited during homogenization by the addition of phenylthiourea and ascorbic acid to ddH_2O. This treatment was otherwise handled identically to the first treatment. Control diets not containing virus were also prepared for both treatments.

The experiment was replicated three times with 20 insects per treatment. The concentrations of protein and free amines in fresh foliage were determined colorimetrically with Coomassie blue reagent (Snyder and Desborough, 1978; bovine serum albumin as standard) and ninhydrin (Rosen, 1957; leucine as standard), respectively. Eight leaflet samples were taken for each analysis. Rutin and chlorogenic acid concentrations were determined colorimetrically with diphenylborate reagent (Broadway et al., 1986) on four 10-mg aliquots of dried, homogenized foliage from each treatment.

RESULTS

Amino Acid Analyses. The incubation of OBs with chlorogenic acid and polyphenol oxidase did not affect the composition of most amino acids of NPV protein (Table 1). A large reduction (>50%) in cysteine content occurred in the PPO/CHA-treated OBs with smaller decreases (ca. 7–9%) in lysine, histidine, and tyrosine.

Dissolution Kinetics. Untreated OBs reached maximal solubility in approximately 30 min in DAS buffer (Figure 1). The PPO/CHA-treated OBs reached maximal solubility in 15 min but were far less soluble compared to untreated OBs throughout the time course of the experiment. The treated OBs were only ca. 30% as soluble as untreated ones at the end of 30 min. Longer incubations did not yield greater dissolution of the untreated or treated OBs. These reductions in solubility are not due to inhibition of OB proteases because PPO and CHA do not reduce proteolytic activity of the solubilized OB at the levels tested (unpublished data).

Digestibility of OBs. At a ratio of CHA to virus of 1:10, the digestibility of the OBs was reduced by 7.8% compared to untreated OBs ($P < 0.05$, Student's t test). The digestibility of OBs at higher concentrations of CHA or PPO were not determined because of the insolubility of the treated OB.

Binding of Chlorogenic Acid to HzSNPV. Chlorogenic acid, in the presence of polyphenol oxidase, bound covalently to OBs (Figure 2). The amount bound depended upon the level of CHA substrate. Approximately 1–2 nmol CHA were bound covalently per milligram of virus over the range tested.

The amount of CHA bound to polyhedrin protein depended upon PPO activity (Figure 3). Over 800 nmol CHA was bound per milligram polyhedrin

TABLE 1. EFFECT OF OXIDATION OF CHLOROGENIC ACID ON AMINO ACID CONTENT OF OCCLUSION BODIES OF HzSNPV[a]

Amino Acid	NPV + CHA	NPV + CHA + PPO
Asp	38.08	37.69
Thr	14.96	14.78
Ser	21.16	21.09
Glu	53.05	54.09
Pro	19.98	20.05
Gly	23.95	24.34
Ala	20.73	21.10
Cys	2.66	1.23[b]
Val	18.01	18.13
Met	4.01	3.97
Ile	15.14	15.10
Leu	25.28	25.79
Tyr	10.56	9.55[b]
Phe	14.61	14.06
His	7.49	6.96[b]
Lys	18.20	16.78[b]
Arg	18.47	18.12

[a] Amino acid content expressed as mmol/100 g. CHA = chlorogenic acid. PPO = polyphenol oxidase.
[b] Reduction greater than 5%.

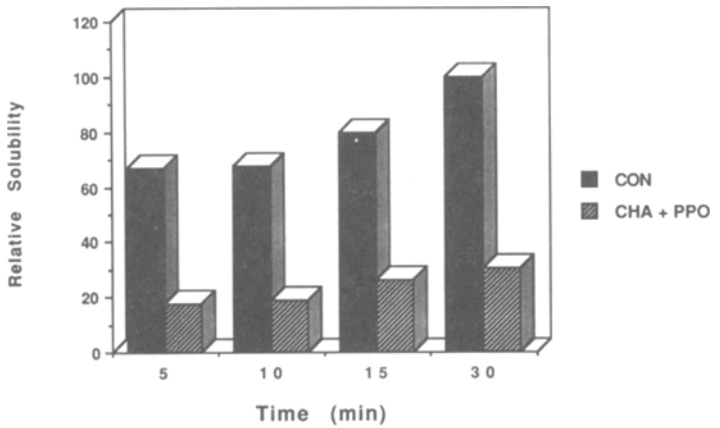

FIG. 1. Rate of dissolution of occlusion bodies of HzSNPV in dilute alkaline saline buffer. CON = control treatment. CHA = chlorogenic acid. PPO = polyphenol oxidase. Each bar represents the mean of three replicates.

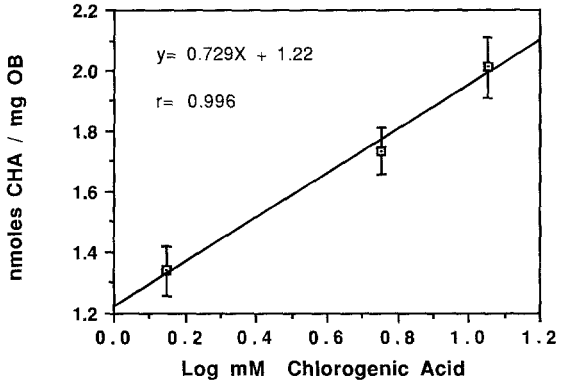

FIG. 2. Covalent binding of chlorogenic acid to occlusion bodies of HzSNPV in the presence of polyphenol oxidase activity. Each point represents the mean of three replicates. Error bars indicate 95% confidence limits. CHA = chlorogenic acid.

at the highest level of PPO activity tested. The amount bound includes both covalently and noncovalently bound CHA.

Effect of Chlorogenic Acid and Polyphenol Oxidase on NPV Infectivity. The incubation of NPV with CHA and PPO significantly reduced viral infectivity (Figure 4). The percent inactivation was highly correlated with log concentration of CHA/OB ($r = 0.935$; $P < 0.01$). The concentration of CHA estimated to give 50% inactivation of NPV infectivity was 6.418 nmol/OB or

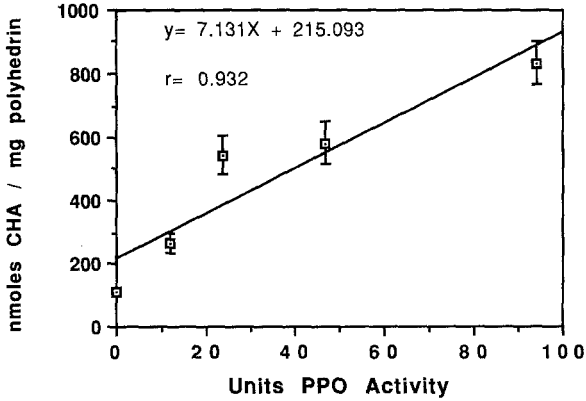

FIG. 3. Effect of polyphenol oxidase activity on binding of chlorogenic acid to polyhedrin protein. Each point represents the mean of three replicates. Error bars indicate 95% confidence limits. PPO = polyphenol oxidase. CHA = chlorogenic acid.

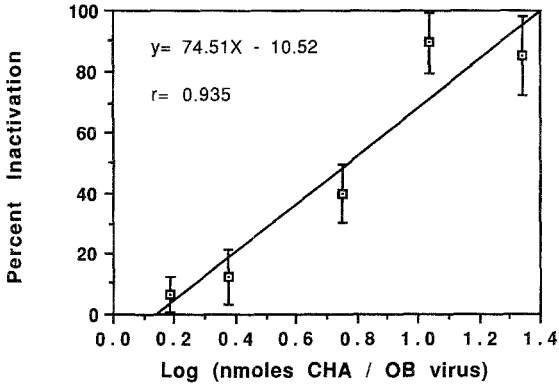

FIG. 4. Effect of chlorogenic acid and polyphenol oxidase on the infectivity of HzSNPV to third-instar *Helothis zea*. One unit PPO activity equals 0.001 change in absorbance at 470 nm/min/ml buffer for chlorogenic acid oxidation. Each point represents the mean of three replicates. Error bars indicate 95% confidence limits. CHA = chlorogenic acid. OB = occlusion bodies.

3.21 μmol/g leaf material containing 500 OB. In fact, the mean concentration of CHA observed in field-grown tomato plants was 3.6 μmol/g leaf (Felton et al., 1987). Mortality in larvae fed untreated OB averaged 45–50%.

Bioassays with Foliage. Inhibition of CHA oxidation significantly increased larval mortality due to HzSNPV (Table 2). The ratio of CHA/OB in these bioassays was 6.88 and yielded 62.5% inactivation when the foliage was allowed to oxidize. Based upon the regression equation generated from bioassays (Figure 4), this concentration would be predicted to result in 52% inactivation. No mortality was observed in control treatments where larvae were exposed to diet devoid of virus.

The foliage used in the bioassays averaged 1.45% protein and 0.67% free amino acids in wet weight. In the treatment with PPO inhibitors, PPO activity

TABLE 2. EFFECT OF OXIDATION OF CHLOROGENIC ACID IN TOMATO FOLIAGE ON INFECTIVITY OF HzSNPV TO THIRD-INSTAR *Heliothis zea*[a]

Treatment	PPO[1]	Rutin	CHA	Mortality (%)
W/ Oxidation	1.30a	0.56a	0.72a	10.0a
W/O Oxidation	0.10b	1.28b	0.93b	26.7b

[a] Means in columns not followed by same letter are significantly different at $P < 0.01$ by Student's t test.

[b] PPO activity expressed as units/mg protein (wet weight). CHA and rutin expressed as μmol/g foliage (wet weight).

was reduced by 92%. The levels of rutin and chlorogenic acid were reduced by 56.3% and 22.6%, respectively, in the treatment with oxidation. Oxidation of phenolics is due primarily to PPO and not to peroxidase because peroxidase activity (measured with guaiacol as peroxidase substrate) was not detected without exogenous hydrogen peroxide. The presence of hydrogen peroxide in the insect gut has not been ruled out as a source for foliar peroxidase activity.

DISCUSSION

The HzSNPV exposed to oxidized chlorogenic acid were less infectious to *H. zea* larvae (Figure 4, Table 2). The infective virions of HzSNPV are embedded in a crystalline proteinaceous occlusion body and must be liberated during alkaline dissolution and/or proteolysis in order to infect larvae (Mazzone, 1985). The OB apparently passes intact through the foregut (pH 6.5–7.0) until reaching the alkaline conditions of the midgut (pH 8.1–9.0), where infective virions are released (Entwistle and Evans, 1985). Tomato PPO activity is very active throughout the digestive system when *H. zea* larvae feed on tomato foliage (Felton et al., 1989), suggesting that substantial binding of CHA to OBs would occur prior to dissolution. In the present study, we did not address the ability of chlorogenoquinone to inactivate infective virions following their release in alkaline conditions.

Our results suggest that decreases in solubility and digestibility of the treated OBs are responsible for reduced infectivity. The deprotonation of polar side groups of tyrosine, lysine, and cysteine occurs under alkaline conditions and initiates changes in OB protein confirmation that lead to dissolution (Rohrmann et al., 1979; Whitt and Manning, 1987; Vlak and Rohrmann, 1985). The binding of chlorogenoquinone to HzSNPV occlusion bodies resulted in decreases in several of these amino acids: lysine, tyrosine, cysteine, and histidine (Table 1). Binding of chlorogenoquinone to these amino acids would interfere with their deprotonation at alkaline pH, thus preventing dissolution.

Chlorogenoquinone binds covalently to HzSNPV, and the amount bound depends upon the levels of CHA substrate and PPO (Figures 2 and 3). The OB is reported to be surrounded by a polysaccharide envelope containing small amounts of amino sugars (Minion et al., 1979). As with amino acids, the $-NH_2$ groups of amino sugars should be susceptible to binding by quinones. However, amino sugars were not detected by our automated amino acid analyses. A much smaller amount of CHA was observed to bind to the intact OB rather than the solubilized polyhedrin protein. This is no doubt due to the larger number of $-NH_2$ and $-SH$ functions exposed in the solubilized polyhedrin proteins.

In the absence of PPO, chlorogenic acid can bind to protein by several mechanisms, including hydrophobic interactions, hydrogen bonding, and co-

valent bonding (Barbeau and Kinsella, 1983; Pierpoint, 1983; McNamus et al., 1983). We observed approximately 100 nmol CHA bound per milligram polyhedrin in the absence of PPO (Figure 3), which may result from both covalent and noncovalent interactions. The pKa's of the phenolic —OH groups of CHA are near 9.0, and at pH 7.0 approximately 3.0% of these groups are ionized (Barbeau and Kinsella, 1983). Thus, under our assay conditions a limited amount of autoxidation occurs via semiquinone radicals forming *o*-quinones. Hydrophobic interactions are favored at a pH near the protein's isoelectric point because of minimal charge repulsions (Barbeau and Kinsella, 1983). The isoelectric point of HzSNPV polyhedrin is 6.08–6.30 (Scharnhorst and Weaver, 1980), therefore, hydrophobic interactions between CHA and polyhedrin would be likely with our assay conditions at pH 7.0. Hydrogen bonding would seem relatively less important because it is favored by acidic conditions (pH < 5.0; Barbeau and Kinsella, 1983).

In general, the binding of quinones to proteins alters the functional properties of proteins in several distinct ways, including reducing protein digestibility (Barbeau and Kinsella, 1985), precipitating protein (Leatham et al., 1980), causing the inter- or intramolecular cross-linking of protein molecules (Stahmann et al., 1977; Motoda, 1979; Matheis and Whitaker, 1984; Cheynier et al., 1988; Cheynier and Van Hulst, 1988; Salgues et al., 1986), or causing oxidative gel formation (Painter and Neukom, 1968). Theoretically, any or all of these mechanisms may interfere with viral infectivity.

The inactivation of the plant viruses, alfalfa mosaic virus and peanut stunt virus, by *o*-quinones has been attributed to "tanning" of viral protein and the formation of disulfide bonds on the protein surface (Mink and Saksena, 1971). The formation of disulfide bonds may interfere with the process of viral uncoating. Our data do not indicate that the quinone formation resulted in an increase in disulfide bonds, although the binding of chlorogenoquinone to the viral OB interfered with OB dissolution and possibly the uncoating process as well. HzSNPV was less susceptible to inactivation by quinones than many plant viruses (Mink and Saksena, 1971; Mink, 1965; Mink et al., 1966; Pierpoint et al., 1977). This may be due to the protective polysaccharide envelope of the occlusion body, which apparently reduces the number of binding sites for chlorogenoquinone on the surface of the OB.

Because other proteins (i.e., dietary protein) are relatively more susceptible to quinone binding (Felton et al., 1989; unpublished data), the possibility exists that dietary protein may "spare" adverse effects on viral infectivity. In order to rule out potential sparing, our bioassays contained casein, which is readily alkylated by the quinone (unpublished data). The addition of this protein during the treatment of virus with PPO and chlorogenic acid did not prevent viral inactivation. Preliminary evidence with another protein, bovine serum albumin, also supports these data. In fact, the presence of protein may enhance

the inactivation, possibly through cross-linking of viral OB with other protein molecules (unpublished data). Correspondingly, significant viral inactivation occurred when NPV was tested with tomato foliage, which contains many molecules that may bind to quinones: proteins, peptides, free amino acids, polyamines, nucleic acids, amino sugars, etc.

Our findings may lead to design and development of chemical and molecular techniques to offset the deleterious effects of phenolics on viral infectivity. The addition of PPO inhibitors, quinone trapping reagents, and/or reducing agents to viral spray formulations may protect the virus from quinone inactivation. Alternatively, genetic variants of NPV containing fewer potential sites for quinone binding could be obtained. Genetic removal of PPO from crop plants to prevent loss of NPV infectivity seems inadvisable because PPO activity in leaf tissue has also been linked to resistance against phloem-feeding insects (Ryan et al., 1982; Duffey, 1986), leaf-chewing insects (Rhoades, 1977; Felton et al., 1989; Duffey and Felton, 1989), phytopathogenic fungi (Gentile et al., 1988; Ampomah and Friend, 1988; Friend, 1979, 1981), nematodes (Brueske and Dropkin, 1973; Hung and Rohde, 1973), and viruses (Mink, 1965; Pierpoint, 1977; Mink et al., 1966).

Aside from lending insights into the improvement of viral efficacy against pest species, our data have heuristic value to ecological theory of insect–plant interactions. An integral component of plant defense theory (Rhoades and Cates, 1976; Feeny, 1976; Coley et al., 1985) is that reduced growth rates of herbivores should enhance the regulatory impact of natural enemies on herbivore populations through increased temporal synchrony of the herbivore and natural enemy. Quinone formation in tomato foliage reduces the growth rate of noctuid herbivores (Felton et al., 1989), but the effectiveness of NPV as a mortality factor may be compromised in plant systems with significant levels of PPO and phenols. In addition to reducing the incidence of viral infection, antibiotic levels of PPO and phenolics may have far greater impact by reducing the number of infective OBs released in the environment. Consequently, the occurrence and severity of viral epizootics may be diminished (Felton et al., 1987; Keating et al., 1988; Anderson and May, 1981). Because of the broad occurrence of polyphenol oxidases and phenolic substrates among plant species (Mayer, 1987; Butt, 1981; Butt and Lamb, 1981; Rhoades and Wooltorton, 1978), our results may be extrapolatable to other host–plant systems. Further study on the impact of these chemical interactions on herbivore populations and their natural enemies is clearly needed.

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FEMALE-PRODUCED OVIPOSITION DETERRENTS OF THE CIGARETTE BEETLE, *Lasioderma serricorne* (F.) (COLEOPTERA: ANOBIIDAE)

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Abstract—Oviposition deterrents of the cigarette beetle, *Lasioderma serricorne*, were isolated from its adult body extract and found to be identical to (2*S*,3*R*,1′*S*)-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1′-methyl-2′-oxobutyl)-4*H*-pyran-4-one (α-serricorone) and its 1′*R*-epimer (β-serricorone) by spectroscopic evidence. Serricorone was previously found as one of the minor sex pheromone components of the same insect, and hence indicating bi-functional nature. The presence of two isomers in the body was proved by careful treatment. Each of them exhibited the same level of oviposition deterring activity, which was less potent than the crude body extract at an increased concentration.

Key Words—Oviposition deterrent, serricorone, cigarette beetle, *Lasioderma serricorne*, Coleoptera, Anobiidae.

INTRODUCTION

Females of certain insects apply some chemicals at oviposition, which inform the conspecific females of the presence of her eggs to avoid overlapping of oviposition. Such female-produced oviposition-deterring pheromones have been demonstrated in bruchid beetles (Yoshida, 1961; Umeya, 1966; Szentesi, 1981), seed weevil (Kozlowski et al., 1983), fruit flies (Prokopy, 1981), and pierid butterflies (Rothschild and Schoonhoven, 1977). Little progress has been made

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in the elucidation of these chemicals; the pheromones have been identified in only two species as yet, the azuki been weevil, *Callosobruchus chinensis* (Oshima et al., 1973), and the European fruit fly, *Rhagoletis cerasi* (Hurter et al., 1987).

The cigarette beetle, *Lasioderma serricorne*, also was observed to use a female-produced oviposition-deterring pheromone, and its adult body extract was revealed to have oviposition-deterring activity (Kohno et al., 1986). In this paper, we report that identification of the oviposition deterrents that were isolated from the adult body extract of *L. serricorne*.

METHODS AND MATERIALS

Insects. Insects used in this study were of the same cultures as described before (Kohno and Ohnishi, 1986). For the extraction of the active components, adults of both sexes that emerged from breeding jars were collected every three to four days. For bioassays, larger females with plump abdomens were collected and then copulated with males on the day prior to the tests. For quantitative and qualitative analyses, each sex was separated at the pupal stage and extracted on the seventh to tenth day after eclosion.

Bioassay. The bioassay method for oviposition-deterring activity was almost the same as described previously except for the assay dose (Kohno et al., 1986; experiment 3). The disk composites, which were composed of three black muslin fabric disks (6 mm in diameter) each, were pinned at four equidistant points around the circumference (2 mm apart from the edge) of a corrugated cardboard disk that was fitted into a Petri dish (6 cm ID). One pair of the pinned disk composites was treated with 10 μl of a hexane solution of the test sample (treated) and the other pair was treated with 10 μl of hexane (control). After evaporation of the solvent, one gravid female was released onto the dish and covered with a 32-mesh metal screen. Under this test condition, females would lay their eggs between the muslin disks. The tests were conducted for 15–18 hr in the dark, 28°C, and 60% relative humidity conditions. The oviposited eggs on each disk were counted and calculated as the oviposition activity index (OAI) according to the following equation:

$$OAI = (T - C)/(T + C)$$

where T and C are the numbers of eggs deposited on the treated composites and the control composites, respectively. When the total number of deposited eggs was less than 10, the data were discarded. The tests were replicated until five sets of data were obtained. The bioassays were carried out at the dose of five female equivalents (FE) in every purification step of the active components.

Instrumentation. High-performance liquid chromatography (HPLC) was

performed with the following two systems: a Hitachi 655 constant-flow pump equipped with a 10 × 300-mm YMC A-024 SIL column, monitoring with a Shodex SE-31 RI detector for purification, and a Shimadzu LC-6A (pump), SPD-6A (UV detector), SCL-6A (system controler), and C-R4A (integrator) system, equipped with a 4.6 × 250-mm Zorbax SIL column for quantitative analysis. Gas chromatography (GC) was performed using a Shimadzu GC-9A with FI detector, equipped with a 0.32-mm-ID × 25-m Carbowax 20 M capillary column. The column temperature was programmed at 2°C/min from 100 to 210°C. Electron impact mass spectra (MS) were measured on a Hitachi M-80 operated at 70 eV. Proton NMR spectra were obtained with a Bruker AM 500 NMR spectrometer using $CDCl_3$ and TMS as solvent and an internal standard, respectively. CD spectra were recorded on a JASCO J-20 automatic spectropolarimeter.

Chemicals. β-Serricorone [(2*S*,3*R*,1′*R*)-serricorone], $[\alpha]_D$ − 269 ± 4 (*c* = 0.11, $CHCl_3$), which was synthesized stereoselectively (Ebata and Mori, 1987), was kindly supplied by Dr. K. Mori of the University of Tokyo, and used for the chemical identification.

The α- and β-serricorone used for the bioassays were synthesized as a diastereomeric mixture (Chuman et al., 1983) and were separated by HPLC under the same conditions as those for purification of the natural serricorones.

Isolation and Identification of Oviposition Deterrents. A total of 1.8 kg of adults (ca. 1,100,000 adults of both sexes) were extracted with hexane for 16 hr at room temperature. After removing the solvent by evaporation, the extracts (99 g) were purified as shown in Figures 1 and 2. Extracts were dissolved in ether and washed with saturated $NaHCO_3$ to remove the acidic components. The active ether phase (89 g) was fractionated three times on a silica gel (Kieselgel 60) column, changing the ratio of ether to hexane stepwise. The most active fraction of the third column chromatography (60% ether–hexane fraction: 3 g) was further purified by three subsequent HPLC separations with 15% (first), 12% (second), 10% (third) ethyl acetate–hexane as the mobile phases, respectively. These separations furnished two active components (A and B) as syrups (A, fraction 2: 2 mg; B, fraction 3: 2 mg). The structures of these two components were investigated by MS, proton NMR, and CD.

Quantitative and Qualitative Analyses of Serricorones. Contents of serricorones in the adult bodies of each sex were analyzed by GC using methyl myristate as an internal standard. Analytical materials were prepared as follows. Male adults were extracted with hexane for 16 hr at room temperature on the seventh to tenth day after eclosion. The copulating 6- to 9-day-old female adults picked from the mixed population were extracted on the next day in the same manner.

The ratio of α- to β-serricorone was analyzed by HPLC using 10% ethyl acetate–hexane as the mobile phase. These components were detected with UV

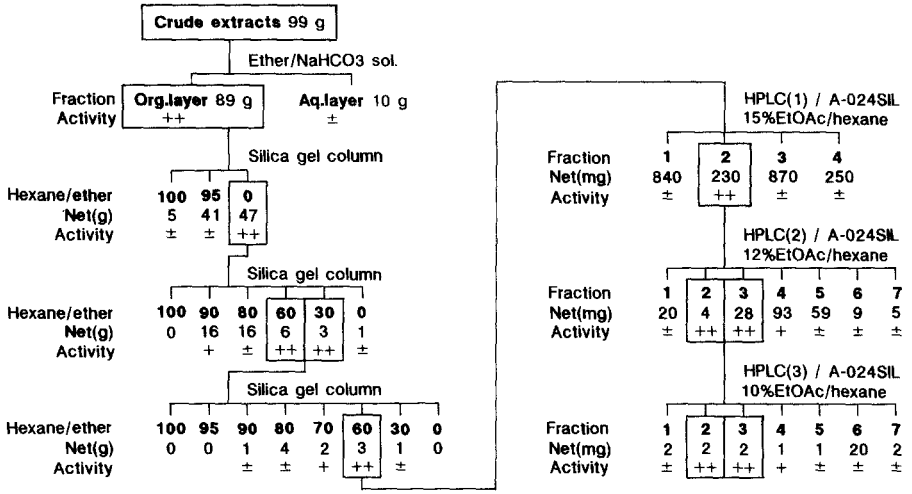

FIG. 1. Purification scheme of oviposition deterrents from adult body extracts. Sign of activity represents following range of oviposition activity indices (OAI) at 5 FE. ±: $+0.2 > \text{OAI} > -0.2$; +: $-0.2 \geq \text{OAI} > -0.4$; ++: $-0.4 \geq \text{OAI}$.

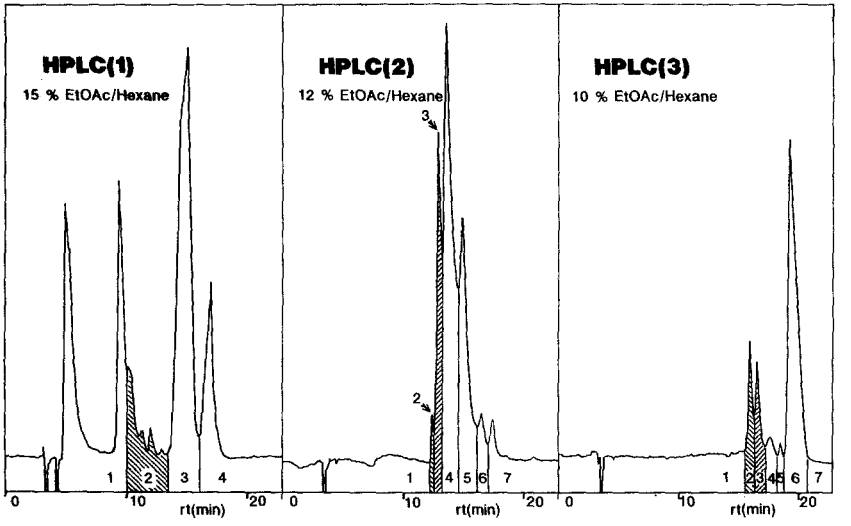

FIG. 2. Three sequential high-performance liquid chromatograms. (see Figure 1). Column: A-024 SIL (10 × 300 mm), flow rate: 5 ml/min.

(254 nm) at the proper retention times as previously tested by the authentic samples. In this analysis, the insects were dipped in hexane for 5 min at room temperature, and the material was subjected to HPLC analysis immediately after removal of the solvent under nitrogen to avoid epimerization.

RESULTS

Identification of Active Components A and B from Adult Body Extract. Mass spectra of the two active components A and B were virtually identical to that of the authentic β-serricorone, with intense ions at *m/z* 43 (26), 57 (83), 113 (100), 124 (39), 182 (82), 238 (M^+, 10).

The proton NMR spectrum of component B [δ; 500 MHz, $CDCl_3$; 0.95 (3H, t, J = 7.4 Hz), 1.02 (3H, d, J = 7.3 Hz), 1.06 (3H, t, J = 7.3 Hz), 1.32 (3H, d, J = 7.0 Hz), 1.48–1.57 (1H, m), 1.79 (1H, m), 1.79 (3H, s), 2.38 (1H, d q, J = 3.0, and 7.3 Hz; H-3), 2.38 (1H, d q, J = 17.8, and 7.3 Hz; H-3′ab), 2.47 (1H, d q, 17.8, and 7.3 Hz; H-3′ab), 3.65 (1H, q, J = 7.0 Hz), 4.12 (1H, d d d, J = 3.2, 5.3, and 8.6 Hz)] was also identical to that of the authentic β-serricorone.

Retention times of components A and B on capillary GC (Carbowax 20 M) were identical to that of synthetic β-serricorone. Components A and B were separated on HPLC (see Figure 4C) and the retention time of component B was identical to that of the authentic β-serricorone.

The proton NMR spectrum of component A was similar to that of the authentic β-serricorone [δ; $CDCl_3$; 0.97 (3H, t, J = 7.4 Hz), 1.03 (3H, d, J = 7.4 Hz), 1.07 (3H, t, J = 7.2 Hz), 1.29 (3H, d, J = 6.9 Hz), 1.45–1.55 (1H, m), 1.80 (1H, m), 1.80 (3H, s), 2.36 (1H, d q, J = 3.1, and 7.4 Hz; H-3), 2.39 (1H, d q, J = 17.6, and 7.2 Hz; H-3′ab), 2.52 (1H, d q, J = 17.6, and 7.2 Hz; H-3′ab), 3.66 (1H, q, J = 6.9 Hz), 4.11 (1H, d d d, J = 3.1, 5.4, and 8.5 Hz)]. J_{H2-H3} values in proton NMR (3.1 and 3.2 Hz in components A and B, respectively) suggested that components A and B had an identical cis configuration at C-2 and C-3. Signals at H-3′ab suggested that component A was the C-1′ epimer of β-serricorone.

To confirm these absolute configurations, CD spectra of components A and B were measured. These results are shown in Table 1. The CD spectral data of component B were in good accord with those of the authentic β-serricorone. Thus the absolute configuration of component B was assigned as 2*S*,3*R*,1′*R*. The CD spectral data of component A showed values with the opposite sign to those of β-serricorone in the 250- to 300-nm region, although it showed values with the same sign in the 350-nm region. The same difference in the CD data has been observed between (2*S*,3*R*,1′*R*)-stegobinone and its 1′*S* epimer, which

TABLE 1. CD SPECTRAL DATA OF COMPONENTS A, B, AND SYNTHETIC OPTICALLY ACTIVE β-SERRICORONE[a]

Wavelength (nm)	Δε		
	Component A	Component B	β-serricorone[b]
355	−1.3	−0.62	−0.54
342	−3.7	−1.2	−0.84
331	−3.9	−0.99	−0.54
289	+22	−14	−13.7
259	−21	+9.0	+8.95

[a] Solvent: hexane, l = 1 mm, 23°C.
[b] Reproduced from Ebata and Mori (1987).

are closely related to serricorones (see Figure 3) (Hoffmann et al., 1981). That is to say, the C-1′ asymmetric carbon has a great effect on the sign of the CD spectra in the 250- to 300-nm region. Consequently, component A is indicated to be 1′*S* epimer of β-serricorone.

In conclusion, structures of components A and B were elucidated to be (2*S*,3*R*,1′*S*)-2,3-dihydro-3,5-dimethyl-2-ethyl-6-(1′-methyl-2′-oxobutyl)-4*H*-pyran-4-one (α-serricorone) and its 1′*R* epimer (β-serricorone), respectively (Figure 3).

Serricorone Content in Adult Body Extract. The α- and β-serricorone have an identical retention time on GC. The combined amount of α- and β-serricorones was determined to be 8.24 ng/FE by GC analysis. The ratio of α- to β-serricorone was found to be 44.8:55.2 by HPLC analysis (Figure 4). No detectable amount of serricorones was observed in the male.

Oviposition-Deterring Activity of Serricorones. OAI of synthetic α- and β-serricorone (2,3-*cis* diastereomeric mixture) and their mixture of (α:β = ca.

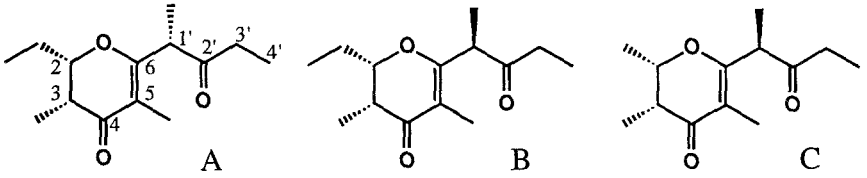

FIG. 3. The α- and β-serricorones and stegobinone. (A) α (2*S*,3*R*,1′*S*)-serricorone; (B) β (2*S*,3*R*,1′*R*)-serricorone; (C) (2*S*,3*R*,1′*R*)-stegobinone.

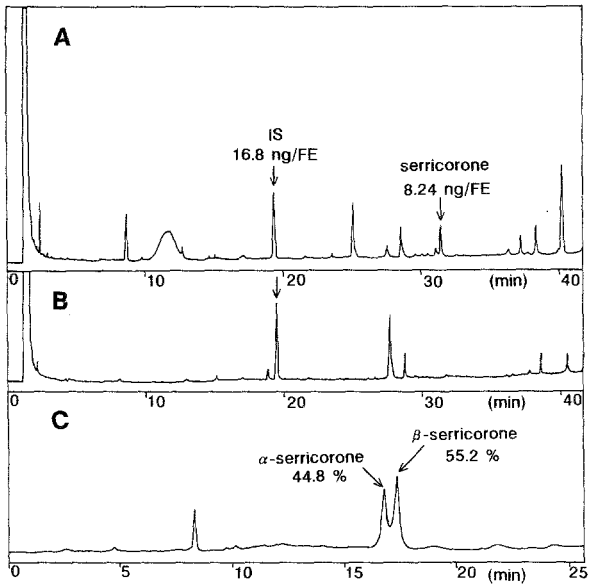

FIG. 4. Serricorone content was estimated at 8.24 ng/FE comparing GC-FI detected peak area with an internal standard (methyl myristate) 16.8 ng/FE, which was added to the extract (A).

In 10 males' extract, a serricorone peak was not detected by the same methods (B). Column: Carbowax 20 M (0.32 mm × 25 m); temperature: 100–210°C at 2°C/min. The ratio of α- to β-serricorone in the female body was estimated to be 44.8:55.2 by UV (254 nm) absorption using HPLC (C). Column: Zorbax-SIL (4.6 × 250 mm); liquid phase: 10% EtOAc–hexane, flow rate: 1.6 ml/min.

1:1) was tested at doses of 0.1, 1, 10, 100, and 1000 ng (0.012, 0.12, 1.2, 12, and 120 FE, respectively). The results are summarized in Table 2. At a dose of 1 ng, α- and β-serricorones revealed oviposition-deterring activity. They showed no significant differences on OAI values at any doses, and when they were mixed, no synergistic and inhibitory effects were observed at any dose.

OAI values of the mixture were compared with those of the crude adult body extract on the FE scale (Figure 5). The threshold level of oviposition-deterring activity of serricorone was below 0.12 FE, which was about 10 times lower than the crude body extract. Even 120 FE of synthetic serricorone could not deter oviposition totally, whereas at a dose of 5 FE, the crude extract deterred it almost completely.

TABLE 2. OVIPOSITION ACTIVITY INDICES (OAI) OF SERRICORONES

Isomer	Mean OAI ± SEM[a]				
	0.1 ng	1 ng	10 ng	100 ng	1000 ng
α	+0.15 ± 0.08a	−0.26 ± 0.18abcd	−0.58 ± 0.12cd	−0.28 ± 0.25abcd	−0.39 ± 0.16bcd
β	−0.06 ± 0.16ab	−0.34 ± 0.14bcd	−0.27 ± 0.13abcd	−0.46 ± 0.20bcd	−0.56 ± 0.07cd
Mixture (1:1)	−0.05 ± 0.13ab	−0.21 ± 0.10abc	−0.51 ± 0.11bcd	−0.66 ± 0.13cd	−0.73 ± 0.06d

[a]Mean OAI and SEM followed by the same letter do not differ significantly ($P < 0.05$) according to Duncan's multiple-range test.

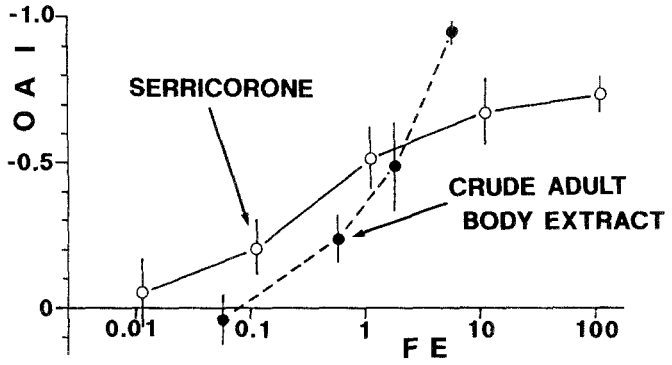

FIG. 5. Oviposition activity indices (OAI) of serricorone (1 : 1 mixture of α and β) and crude body extract. Mean (circle) with bar representing standard error of the mean (N = 5).

DISCUSSION

Serricorone was first described as one of the minor sex pheromone components of this species (Chuman et al., 1983) and was reported to have an aphrodisiac effect and little attractivity for the male cigarette beetle (Chuman et al., 1985). In this paper we have shown that serricorone possesses an oviposition-deterring activity as an additional function. Such a phenomenon was also reported in the fruit flies—*Rhagoletis pomonella* (Prokopy and Bush, 1972), *R. cerasi* (Katsoyannos, 1975), and *Ceratitis capitata* (Prokopy and Hendrichs, 1979)—whose oviposition-deterring pheromones showed male arrestant activity. Although the oviposition-deterring pheromone of *R. cerasi* was purified and identified recently (Hurter et al., 1987), the purified pheromone has not been determined to act as the sex pheromone. Therefore, it is still possible that the male arrestant activity to oviposited substrates of the fruit flies is attributable to substances other than the oviposition deterring pheromones. Serricorone is the first pheromone to be determined at the molecular base to act bifunctionally as a sex pheromone and an oviposition deterrent.

The absolute configuration of natural serricorone was confirmed by comparison of CD spectral data of the natural pheromone with those of synthetic optically active (2*S*,3*R*,1′*R*)-serricorone: the natural product was indicated to be composed of an epimeric mixture of (2*S*,3*R*,1′*R*)- and (2*S*,3*R*,1′*S*)-serricorones (Ebata and Mori, 1987). Since C-1′ of the serricorone is easily epimerized, it was still ambiguous whether the insect produces these two epimers in the body or if one is derived from the other under the experimental conditions. By careful treatment to avoid epimerization in the isolation procedure, we proved that neither of these two epimers is an artifact: both are produced in the female body.

Stegobinone, a component of the sex pheromone of the drugstore beetle (*Stegobium paniceum*) (Kuwahara et al., 1978), is also epimerized easily to furnish a C-1′ epimer, which inhibits the pheromonal activity of the natural pheromone (Kodama et al., 1987). In contrast, α- and β-serricorone and their mixture had the same levels of oviposition-deterring activity; even when epimerization occurs, no additional effect is produced. These facts well account for the difference in the effective life periods required for a sex pheromone or an oviposition-deterring pheromone; minutes or hours for the former and days or weeks for the latter are needed for them to function in their respective biological activities.

The α- and β-serricorones elicited a higher oviposition-deterring activity at doses below 1.2 FE than the crude adult body extract, but the dose–response curve of serricorone did not increase linearly with an increase of the dose above 1.2 FE. Serricorone could not deter oviposition completely even at 120 FE, which was 20 times as high as the completely deterring dose of the crude body extract. It may be due partly to saturation of the serricorone vapor in the small assay dishes, or it may indicate the presence of other unknown active or synergistic component(s) in the body extract.

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A MUTATION IN PHEROMONAL COMMUNICATION SYSTEM OF CABBAGE LOOPER MOTH, *Trichoplusia ni*

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Abstract—A mutation that results in a dramatic change in the relative proportions of the pheromone components produced by female cabbage looper moths has been found. The most notable changes involve reduction in the emission rate of the major component [(Z)-7-dodecenyl acetate], near absence of a component [(Z)-5-dodecenyl acetate] that is normally present at about 20% of the major component, and a remarkable ca. 20-fold increase in a trace component [(Z)-9-tetradecenyl acetate]. In spite of the multiplicity of changes in the pheromone blend, a genetic analysis indicates that the condition is controlled by a single autosomal gene. These mutants are ineffective in attracting conspecifics, but do attract another distantly related noctuid moth. These results suggest that the evolutionary process that leads to distinct chemical signals in sibling species may include single gene mutations that lead to major changes in the species-specific blend.

Key Words—Sex pheromone, genetics, reproductive isolation, (Z)-7-dodecenyl acetate, (Z)-5-dodecenyl acetate, (Z)-9-tetradecenyl acetate, Lepidoptera, Noctuidae, *Trichoplusia ni*, cabbage looper moth.

INTRODUCTION

Speciation in animals depends on the evolution of effective reproductive barriers between populations, which often include specificity of the communication system between the sexes. Mutations in genes controlling the signal and the response create the variation upon which selection must act before divergences in communication can occur. In moths the specificity of the chemical communication system often depends on the blend of pheromonal components released

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by females (Klun et al., 1979; Roelofs and Cardé, 1974; Tumlinson et al., 1974). Interpopulational variation in the blend ratio of pheromone components emitted by females and a correlated behavioral response specificity in males have been well documented within a few species (Bailey et al., 1988; Klun and cooperators, 1975; Löfstedt et al., 1986; Roelofs et al., 1985, 1987).

The sex pheromone communication system of the cabbage looper moth, *Trichoplusa ni*, has been studied extensively, both in terms of the compounds emitted by females (Berger, 1966; Bjostad et al., 1980a,b, 1984; Haynes and Hunt, 1990) and the behavioral responses of males to blends of these compounds (Landolt and Heath, 1987; Linn and Gaston, 1981; Linn et al., 1984, 1988). We have discovered a mutation in the cabbage looper moth that results in a dramatic change in the pheromone blend released by females. Studies of this type of mutation may increase our understanding of the evolutionary process by which effective communicatory barriers may arise, and they may serve as a valuable probe in studies of the biosynthetic pathways of pheromones.

METHODS AND MATERIALS

Insects. The laboratory colony in which the abnormal females were discovered originated from a field population near Riverside, California. This colony has been maintained using the procedures of Shorey and Hale (1965) for at least 10 years without the introduction of any individuals from field populations. Three individuals that produced excessive quantities of one of the pheromone components, (Z)-9-tetradecenyl acetate (Z9–14 : Ac), which departed dramatically from the normal range of variation observed in females from field populations from across the United States (Haynes and Hunt, 1990), were discovered during the course of collecting volatiles from females from this laboratory population. One of these individuals was successfully mated, and her offspring were used for individual sib–sib matings. Over successive generations, a colony was established in which all females expressed the abnormal phenotype. A normal colony was also established in which no individuals expressed the abnormal phenotype. These two colonies served as sources for individuals of known phenotypes for genetic analyses and for field studies.

Pupae from these two colonies or from other crosses were separated according to sex. Female pupae were housed in 1-pint (473 ml) paper cartons with nylon-screen lids. Emerged adults were removed daily and transferred to additional cartons. Just prior to the initiation of the scotophase when females were 3–5 days old, they were placed individually in 4-cm-diameter × 4-cm-high hardware cloth (3-mm mesh) cages closed with aluminum lids.

Analytical Procedures. Collection of pheromone from females followed the procedures described by Haynes and Hunt (1990), who used a method mod-

ified from Baker et al. (1981). During the second through eighth hour of the scotophase, a period during which calling behavior was observed under the experimental conditions, a female was removed from an environmental chamber and inserted abdomen first into a glass female-holder. The glass holder was 5.5 cm long, 0.6 cm in diameter, and had a ca. 1-mm hole at the distal end that allowed the ovipositor and associated sex pheromone gland to extrude out of the holder when pressure was applied to the head of the female with a pipe cleaner. The holder was then inserted into the all-glass collector described by Haynes et al. (1984). A stream of nitrogen gas flowed through the collector at a rate of 120 ml/min. After the 10-min collection period, 10 ng of an internal standard [(*E*,*Z*)-4,7-tridecadienyl acetate] was added to the adsorbent glass wool surface at the distal end of the collector, and then the collector was rinsed out with two 50-μl aliquots of CS_2. This volume was reduced to ca. 1 μl before it was injected into a 30-m Carbowax 20 M column housed in a Hewlett-Packard 5890A GC linked to a Hewlett-Packard 5970B Mass Selective Detector (MSD). After sample injection, the column temperature was held at 80°C for 2 min, then was increased at a rate of 10°C/min to 200°C. The sensitivity of this instrument was optimized by manual tuning for *m/z* ion 69 of the tuning standard perfluorotributylamine combined with selective ion monitoring of *m/z* ions 54, 55, and 67, which are in high abundance in all of the reported pheromone components. Standard curves were established that related peak area (*m/z* 54 + 55 + 67) to the mass of each pheromone component. These standard curves were critical because the relative abundance to these ions was not the same for all molecules of interest. The lower analytical limit of this technique was ca. 0.01 ng.

For a pooled sample of volatile collections from individuals of the mutant type, the procedure of Buser et al. (1983) was utilized to confirm the position of the double-bond of the compound corresponding in retention time to Z9–14 : Ac. After the dimethyl disulfide adducts were prepared, they were analyzed using the same capillary column listed above. The temperature program was modified in that the column was held at 120°C for 2 min, then the temperature was increased by 20°C/min to 250°C. The MSD was operated in the scan mode sampling from 40 to 350 amu. Mass spectra and retention times were compared to those found in the dimethyl disulfide adducts of authentic Z9–14 : Ac.

Field Tests. Pherocon 1C traps were baited with two caged females from either the normal colony or the mutant colony. Traps were placed at least 20 m apart in soybean fields, alfalfa fields, a community vegetable garden, and a suburban backyard. The openings of the traps were approximately 10–20 cm above the plant canopy. Traps were deployed in a randomized block design, and the number of moths captured was determined daily. Traps were refitted with new sticky trap bottoms, and their positions were rerandomized after each night. This experiment was run between August 17 and August 29, 1988. The

results include 35 blocks of the experiment in which moths were captured in the traps.

RESULTS AND DISCUSSION

Using capillary gas chromatography of volatiles emitted by females, we quantified the differences in the pheromone blends of mutant and normal females (Figure 1). While significant differences were determined in the quantities of

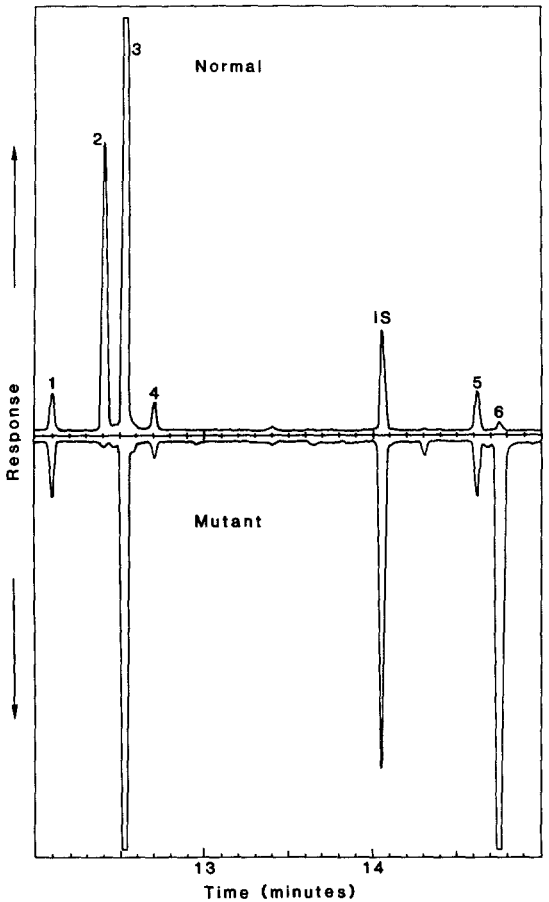

FIG. 1. Capillary GLC traces of sex pheromone components emitted by a normal (upper trace) and a mutant female (lower trace). Pheromonal components are dodecyl acetate (1), (Z)-5-dodecenyl acetate (2), (Z)-7-dodecenyl acetate (3), 11-dodecenyl acetate (4), (Z)-7-tetradecenyl acetate (5), and (Z)-9-tetradecenyl acetate (6). An internal standard (IS), (*E*,*Z*)-4,7-tridecadienyl acetate was used in analyses.

TABLE 1. EMISSION RATES OF SIX PHEROMONE COMPONENTS FROM NORMAL AND MUTANT CABBAGE LOOPER MOTHS, *Trichoplusia ni*

Pheromone component	Quantity emitted (ng/10 min) ± SEM[a]		Ratios of quantities (mutant/normal)
	Normal (N = 68)	Mutant (N = 125)	
12:Ac	3.8 ± 0.31*	3.2 ± 0.22*	0.84
Z5-12:Ac	9.2 ± 0.71**	0.29 ± 0.06**	0.03
Z7-12:Ac	53.1 ± 4.6**	18.3 ± 1.29**	0.34
11-12:Ac	1.8 ± 0.15**	0.65 ± 0.05**	0.36
Z7-14:Ac	0.82 ± 0.07**	0.39 ± 0.02**	0.48
Z9-14:Ac	0.47 ± 0.05**	9.4 ± 0.71**	20.0

[a] Normal and mutant moths are significantly different: $*P < 0.05$ and $**P < 0.0001$; Kruskal-Wallis one-way analysis of variance.

all of the pheromonal components, the most obvious differences were that the mutant females emitted only 3% as much (Z)-5-dodecenyl acetate (Z5-12:Ac) as normal females and that they produced 20 times as much Z9-14:Ac as normal females (Table 1). In some individuals Z9-14:Ac was more abundant than Z7-12:Ac. The identity of Z9-14:Ac was confirmed by a mass spectrometric analysis of dimethyl disulfide derivatives of volatiles collected from mutant females.

The documented changes in the pheromone blends of these mutant females were critical to the effectiveness of the communication system, since traps baited with mutant-type females caught significantly fewer conspecific males than normal females (Table 2). In addition, the specificity of the captures of males was affected. Traps baited with mutant females captured significantly more male

TABLE 2. CAPTURES OF MALE CABBAGE LOOPER MOTHS (CL), *Trichoplusia ni*, AND MALE BLACK CUTWORM MOTHS (BCW), *Agrotis ipsilon*, IN TRAPS BAITED WITH 2 VIRGIN FEMALE *T. ni.* OF NORMAL OR MUTANT PHEROMONE PHENOTYPES[a]

Traps baited with	Mean no. of males caught/trap/night ± SEM	
	CL	BCW
Normal (N = 35)	2.66 ± 0.46	0.03 ± 0.02
Mutant (N = 35)	0.06 ± 0.04	0.57 ± 0.19

[a] Replicates of the experiment were run in a soybean field, an alfalfa field, a community vegetable garden and a suburban back yard. Unbaited control traps caught no males of either species. Means in columns are significantly different according to Kruskal-Wallis analysis of variance.

black cutworm moths, *Agrotis ipsilon*, than did normal females. The pheromone blend of *A. ipsilon* has been identified as a 5 : 1 blend of (Z)-7-dodecenyl acetate (Z7–12 : Ac) to Z9–14 : Ac (Hill et al., 1979). Therefore, it should not be surprising that males of this species responded to mutant female *T. ni*, since these females produced a blend ratio of Z7–12 : Ac/Z9–14 : Ac that was similar to the blend released by female *A. ipsilon*.

Inheritance of the pheromone mutation is most likely controlled by a single recessive autosomal gene (Table 3). F_1 crosses between mutant and normal lines resulted in all normal offspring, regardless of whether the female was from normal or mutant lines. In Lepidoptera the male is the homogametic sex (Robinson, 1971), therefore a sex-linkage hypothesis would predict all mutant offspring from a parental male from a pure mutant line. This pattern was not observed. These results also indicate that the inheritance of this gene may be recessive, since the mutant phenotype was not expressed in the F_1 offspring. This hypothesis was further supported by F_2 results, in which the observed phenotypic ratio was not significantly different from a 3 : 1 ratio (normal to mutant) predicted by the hypothesis of recessive inheritance. Backcrosses of F_1 offspring to the parental mutant type yielded a phenotypic ratio that was not significantly different from a 1 : 1 ratio. The results of these experiments would be predicted by autosomal inheritance of a recessive gene.

A single gene mutation resulted in the multiplicity of changes that were observed in the pheromone blend. Recently, it was demonstrated that pheromone components in *T. ni* and other species are produced by four enzymatic steps from available precursors (Bjostad et al., 1984; Bjostad and Roelofs, 1983). These steps include delta-11-desaturation, chain-shortening, reduction, and acetylation. A change in the specificity or relative quantity of any one of

TABLE 3. CROSSES TO DETERMINE GENETIC BASIS OF INHERITANCE OF MUTANT PHENOTYPE[a]

Cross (number of crosses)		Observed ratio of phenotypes (N : M)	Expected ratio of phenotypes (N : M)
Parental	M × M (8)	0 : 111	All mutant
Parental	N × N (7)	76 : 0	All normal
F_1	M♀ × N♂ (4)	44 : 0	All normal
F_1	N♀ × M♂ (5)	55 : 0	All normal
F_2	MN × MN (13)	147 : 48	3 : 1 (146.25 : 48.75)
Backcross	MN × M (13)	87 : 93	1 : 1 (90 : 90)
Backcross	MN × N (6)	66 : 0	All normal

[a] Observed ratios of female phenotypes are compared to predicted ratios resulting from recessive inheritance of a single autosomal gene. M = mutant phenotype; N = normal phenotype; MN = F_1 offspring (heterozygote).

the enzymes involved in pheromone biosynthesis could result in a change in the blend ratios of the pheromone components. Alternatively, a mutation in a single regulatory gene could influence the abundance of several of these enzymes. One cannot argue that the decrease in Z5–12 : Ac is directly related to the observed increase in Z9–14 : Ac, because the two pheromonal components are produced from different biosynthetic precursors. In contrast, the observed decrease in emission of Z7–12 : Ac may be more directly related to the increase in Z9–14 : Ac since the fatty acyl precursors of these two compounds are separated by only one cycle of chain-shortening. Studies of this pheromone mutation should provide a effective tool for further investigations into the biosynthetic pathways of pheromones. In addition, because the mutation seems to have occurred at a single locus, further study should provide information about how a single gene product can regulate the species specificity of pheromone blends.

Clearly the mutation in the female-produced pheromone represents the type of change that is necessary before speciation can occur. However, under natural conditions in the field, females that express the mutant pheromone phenotype would be at a tremendous reproductive disadvantage, assuming that there is no preadaptive genetic linkage between the males' response and the females' blend ratio. In our laboratory mating colonies, this disadvantage disappeared since long-range mate location, during which the specificity of the pheromone blend is of paramount importance (Linn et al., 1988), was no longer required. In order for speciation to occur it would be essential for directional selection on the males' response to be operative. We may be able to select for pheromone-based reproductive barriers by allowing only those males that successfully fly upwind and locate mutant females to mate. Using this selection regime, we expect to gain insight into the special biological and ecological conditions that would be required before speciation is complete.

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EFFECT OF RELEASE RATE AND RATIO OF (Z)-11-HEXADECEN-1-OL FROM SYNTHETIC PHEROMONE BLENDS ON TRAP CAPTURE OF *Heliothis subflexa* (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Response of male *Heliothis subflexa* to pheromone-baited traps containing blends of tetradecanal, (Z)-9-tetradecanal, hexadecanal, (Z)-7-hexadecenal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, hexadecan-1-ol acetate, (Z)-7-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol acetate, (Z)-11-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol, and (Z)-11-hexadecen-1-ol was evaluated. Analysis of trap capture data indicated that (Z)-11-hexadecen-1-ol was a critical component of the pheromone blend. It was determined from emission rate data and measurements of the ratio of pheromone components emitted from rubber septa tested that a significant increase in trap capture of male *H. subflexa* occurred when the blends investigated released the alcohol in a narrow range relative to the total amount of pheromone emitted. The optimum range of release ratio of the alcohol for the capture of males in sticky traps was determined to be 0.9–3.5% of the pheromone blend. This release ratio range was reduced to 0.9–1.6% when bucket traps were used.

Key Words—Pheromone formulation, trap design, insect behavior, pheromone specificity, *Heliothis subflexa*, Lepidoptera, Noctuidae.

INTRODUCTION

Heliothis subflexa is a major pest of cultivated ground cherry (*Physalis* spp.) in Mexico. In the United States, interest in *H. subflexa* is due to the potential use of hybrids that result when *H. subflexa* females and *Heliothis virescens* males are mated (Laster, 1972). The male progeny produced are sterile, and the fertile females continue to produce sterile male offspring. This discovery provides a

potential for population suppression of *H. virescens* by the introduction of sterile hybrid insects into wild populations. A better understanding of the pheromone components of the parent species will compliment ongoing research regarding the biochemistry of pheromone production and provide improved pheromone blends for monitoring *H. subflexa*.

The sex pheromone components found in ovipositor extracts of *H. subflexa* have been reported by Teal et al. (1981) and Klun et al. (1982). These reports did not provide detailed information regarding the optimum pheromone blend for use as a trap bait. Our efforts to utilize the synthetic blends of components previously reported resulted in highly variable captures of *H. subflexa* males. Captures of male *H. subflexa* in traps baited with synthetic pheromone often were significantly lower in numbers compared to traps baited with sexually mature virgin female *H. subflexa*. Recently, Heath et al. (1990) investigated the periodicity of pheromone production and release from sexually mature *H. subflexa* and *H. virescens* females during various times in the scotophase. During this investigation, it was determined that a small percentage of (Z)-11-hexadecen-1-ol was emitted by calling female *H. subflexa*. Based on this, we subsequently investigated the blend of chemicals required for optimum trap catch of *H. subflexa*. We report here the effect that small amounts of (Z)-11-hexadecen-1-ol, emitted over a narrow range, has on the trapping of male *H. subflexa* when added to previously reported sex pheromone components. Capture of male *H. subflexa* in traps baited with pheromone blends containing various ratios of the alcohol also were compared and the optimum release rate of the optimum ratio determined.

METHODS AND MATERIALS

Formulations. Synthetic pheromone blends were formulated on rubber septa (#8153-022, A.H. Thomas Co., Philadelphia, Pennsylvania) that were extracted with methylene chloride for 24 hr and air dried prior to formulation. The percentage by weight of a component added to the blend was calculated on the basis of its relative volatility determined from retention indices on liquid crystal capillary gas chromatographic (GC) columns (Heath and Tumlinson, 1986) and a method developed to predict release ratios of components of a blend from rubber septa (Heath et al., 1986). Each septum was loaded with 100 μl of a hexane solution of the blend pipetted into the well on the large end of the septum. Septa were allowed to equilibrate 48 hr prior to use.

Qualitative and quantitative analysis of material loaded on the septa were obtained using a Hewlett-Packard model 5890 gas chromatograph equipped with splitless capillary injectors and flame ionization detectors. A Nelson 4000 data

system was used for chromatogram storage and analysis. Helium was used as the carrier gas at a linear flow of 18 cm/sec. Columns used for analysis were a 50-m × 0.25-mm-ID Supelco wax (bonded Carbowax) and a 50-m × 0.25-mm CPS-1 high-polarity cyano column. Both columns were operated at 60°C for 2 min, then temperature programmed at 30°/min to 160°C. Both of these columns afforded complete resolution of the previously reported pheromone components. The compounds analyzed included tetradecanal (14:Al), (Z)-9-tetradecanal (Z9–14:Al), hexadecanal (16:Al), (Z)-7-hexadecenal (Z7–16:Al), (Z)-9-hexadecenal (Z9–16:Al), (Z)-11-hexadecenal (Z11–16:Al), hexadecan-1-ol acetate (16:Ac), (Z)-7-hexadecen-1-ol acetate (Z7–16:Ac), (Z)-9-hexadecen-1-ol acetate (Z9–16:Ac), (Z)-11-hexadecen-1-ol acetate (Z11–16:Ac), (Z)-9-hexadecen-1-ol (Z9–16:OH) and (Z)-11-hexadecen-1-ol (Z11–16:OH).

All synthetic material used was obtained from previous research efforts and was greater than 99% pure. When necessary, materials were further purified using high-performance liquid chromatography on a 25 × 2.5-cm (OD) $AgNO_3$ column (Heath and Sonnet, 1980). Confirmation of the identity of the compounds was obtained using GC-mass spectrometry with a Nermag model R1010 mass spectrometer in the chemical ionization mode. The CPS-1 capillary column used in previous analyses was used in the GS-mass spectrometric analyses using helium as the carrier gas. Either methane or isobutane was used as the reagent gas in the mass spectrometer. Release rate and ratio of pheromone emitted was determined on septa aged in the laboratory as well as septa used in the field experiments. Determination of the ratio and rate of release of the pheromone from a septa was performed by placing the septa in a stainless-steel chamber in which purified air was introduced at a wind velocity of 0.225 m/sec over the septa. The volatilized pheromone was collected on a small filter prepared by sealing 50 mg of Super Q80–100 mesh (Alltech Associates, Inc.), between two plugs of glass wool in a 4-cm-long × 4.0-mm (ID) Pyrex tube. The Super-Q filter was placed at the exit end of the stainless-steel chamber and 1 liter/min of filtered air was passed through the system. After 1 hr (2 hr for septa with low concentration) of collection, the filter was removed and rinsed with four aliquots (100 μl) of methylene chloride, the volume concentrated to 5–10 μl, and tridecane was added as internal standard.

Field Tests. Five trapping experiments were conducted near Gainesville, Florida, in fallow fields that contained ground cherry (*Physallis* spp.) infested with *H. subflexa*. Tests were carried out in September and October 1987. Sticky (Zoecon Corp.) and bucket traps (International Pheromone Moth Traps, Merseyside, England) were positioned ca. 1 m above ground on metal stakes. In all experiments, blank traps were used as controls. In experiments conducted with sticky traps, each replicate contained a trap baited with two virgin female *H. subflexa* confined in a wire cage. A 3 × 4-cm piece of No-Pest Insecticide

Strip (Texize, Greenville, South Carolina) was placed in the bottom of bucket traps to kill captured moths. No female-baited bucket traps were used.

In the initial field experiment, five different combinations of synthetic pheromone components were compared with two live females and blanks using sticky traps. All pheromone treatments consisted of a five-component blend (5CB): 16:Al, Z9–16:Al, Z11–16:Al, Z9–16:Ac, Z11–16:Ac in a load ratio of 1.3, 23.3, 52.3, 5.2, and 12.9%, respectively, at a total load of 500 μg/septum. Treatments were: five-component blend (5CB); 5CB + 2% of Z7–16:Ac; 5CB + 3.2% Z11–16:OH; 5CB + 2.0% Z7–16:Ac + 3.2% Z11–16:OH; and 5CB + 2.0% Z7–16:Ac + 1.1% Z9–16:OH + 3.2% Z11–16:OH. Traps were spaced 15 m apart, and treatments were randomized daily for 10 days. Results were analyzed using analysis of variance, and differences among mean percentage of daily trap catch were determined using Duncan's multiple-range test (Duncan, 1955).

In experiment 2, sticky traps baited with a six-component pheromone blend alone or with the six-component blend to which additional components were added were compared with sticky traps baited with two live females and a blank trap. The six-component blend (6CB) consisted of the 5CB with the addition of 1.3% of Z7–16:Ac at a total load of 1 mg. Treatments for experiment two were: 6CB + 1.7% Z9–16:OH; 6CB + 3.1% Z11–16:OH; 6CB + 1.3% Z9–16:OH + 3.1% Z11–16:OH; and the 6CB + 1.1% Z9–16:OH + 3.2% Z11–16:OH + 0.3% *S*-16:Ac + 0.05% 14:Al + 0.04% Z9–14:Al. Three blocks, each containing five different synthetic blends, traps baited with females, and blank traps were randomized daily for five days for a total of 15 replications. Results were analyzed as described above.

The effect of increasing the percentage of Z11–16:OH in synthetic blends on the capture of males was studied using both sticky traps and bucket traps. Septa containing 1 mg of 6CB were used as the basic lure, and treatments included the 6CB plus 1.5, 3.1, 5.8, and 10% (of the total blend) of Z11–16:OH. Blocks testing blends in sticky traps included a trap baited with two virgin female *H. subflexa*. Unbaited traps were used as controls in each test. Three blocks of seven treatments using sticky traps were randomized daily for five days. Similarly, three blocks of six treatments using bucket traps were randomized daily for five days.

The ratio of Z11–16:OH resulting in the greatest catch of male *H. subflexa* in the preceding test was used in a study that compared the effect of concentration of pheromone on capture of males in bucket traps. Septa were loaded with the six-component blend containing 4.0% Z11–16:OH at 0.3, 1, 3, 6, or 10 mg. Three blocks containing the five treatments were randomized over a six-day period. Because of loss of a trap during the sixth night, only 17 replicates were obtained.

RESULTS

In a previous study, Heath et al. (1990) determined that a blend of seven components was emitted by calling virgin females of *H. subflexa*. Compounds emitted were identified as 1.8% 16:Al, 27.6% Z9-16:Al, 60.9% Z11-16:Al, 1.2% Z7-16:Ac, 2.1% Z9-16:Ac, 4.6% Z11-16:Ac, and 1.8% Z11-16:OH. Based on this information, septa were loaded to release compounds under investigation at this ratio (Tables 1 and 2).

Prior to and concurrent with the field tests, septa formulated with various

TABLE 1. ANALYSIS OF RUBBER SEPTA FORMULATED WITH *H. subflexa* PHEROMONE COMPONENTS

Compound	Load ratio	Release ratio	Release rate (ng/hr) ± SD (N = 4) Laboratory,[a] aged 3 days	Field, aged 3 days	Field, aged 7 days	Field, aged 12 days
16:Al	1.3	1.1 ± 0.7	2.2 ± 4.1	3.0 ± 0.3	2.3 ± 0.2	2.4 ± 0.3
Z9-16:Al	22.9	28.9 ± 1.4	56.6 ± 4.0	53.2 ± 11.5	54.5 ± 5.0	50.8 ± 22.5
Z11-16:Al	53.4	61.5 ± 3.0	120.3 ± 10.7	125.6 ± 12.9	116.0 ± 12.0	111.1 ± 11.8
Z7-16:Ac	1.9	0.7 ± 0.4	1.3 ± 0.3	1.0 ± 0.0	1.1 ± 0.1	1.3 ± 0.4
Z9-16:Ac	5.3	2.0 ± 0.9	4.0 ± 2.7	3.7 ± 0.5	3.5 ± 0.2	4.3 ± 1.6
Z11-16:Ac	12.1	4.2 ± 1.9	8.2 ± 4.0	7.5 ± 1.2	6.8 ± 0.5	8.8 ± 3.3
Z11-16:OH	3.1	1.6 ± 0.7	3.1 ± 0.8	2.8 ± 0.4	3.0 ± 0.4	2.6 ± 0.8
Total amount emitted			195.7 ± 12.2	196.8 ± 14.3	187.2 ± 17.8	181.4 ± 16.8

[a] Septa kept in fume hood with wind speed = 0.22 m/sec.

TABLE 2. RELEASE RATIOS OBTAINED FROM ANALYSIS OF FIELD SEPTA USED IN DOSE–RESPONSE[a] (N = 60)

Compound	16:Al	Z9-16:Al	Z11-16:Al	Z7-16:Ac	Z9-16:Ac	Z11-16:Ac	Z11-16:OH
				Percent Release ± S.D.			
Average	1.4 ± 0.4	29.0 ± 1.2	60.8 ± 1.9	0.7 ± 0.3	2.0 ± 0.5	4.4 ± 1.5	1.5 ± 0.6
minimum value	1.0	26.6	56.4	0.3	1.1	2.4	0.6
Maximum value	2.6	31.1	62.5	1.4	2.9	9.0	2.5

[a] Septa load = 0.3, 1, 3, 6, 10 mg and analyzed after 3, 7, and 10 days in field.

blends were analyzed in the laboratory to ensure that the integrity of the formulation had not changed. Examples of results from the analysis of septa loaded with a blend containing 3.1% of the alcohol are shown in Tables 1 and 2. As shown in Tables 1 and 2, septa maintained in a laboratory hood at a wind speed of ca. 0.22 m/sec for three days emitted pheromone at a rate close to that observed from septa that had been exposed in the field for three days. Analyses of septa that were used in field experiments for 7–12 days indicated that the release rate of the total pheromone blend had decreased by 8% during this period compared with release rates obtained from three-day-old septa. These results were typical for all septa used in this study based on the analysis of 120 septa.

Although the release rate of the pheromone from septa decreased with time, the release ratio of the blends remained reasonably constant throughout the test periods. Shown in Tables 1 and 2 are the results of 60 analyses made on septa used in the dose response experiment. These data indicate that minimal variation in release ratio occurred with septa loaded with different amounts of pheromone and exposed in the field for various periods of time.

In experiment 1 (Table 3), significantly higher percentages of male *H.*

TABLE 3. RESPONSE OF MALE *H. sublfexa* TO STICKY TRAPS BAITED WITH FIVE-COMPONENT BLEND[a] (5CB), + ADDITIONAL COMPONENTS AND VIRGIN FEMALES

+Z7-16:Ac	+Z9-16:OH	+Z11-16:OH	+14:Al +Z9-14:Al +16:Ac trap/night	Average number males/ replicate[b]	Mean % males/ captured
Experiment 1 (septa load = 500 μg, $N = 10$)					
–	–	–	–	1	2 a
+	–	–	–	1	1 a
–	–	+	–	14	21 b
+	–	+	–	16	24 b
+	+	+	–	13	18 b
2 Females				24	34 c
Experiment 2 (septa load = 1 mg, $N = 15$)					
+	+	–	–	4	5 a
+	–	+	–	12	22 b
+	+	+	–	18	24 b
+	+	+	+	19	26 b
2 Females				17	23 b

[a] Five component blend = 1.3% 16:Al, 24.3% Z9-16:Al, 54.3% Z11-16:Al, 4.2% Z9-16:Ac, 13.9% Z11-16:Ac.

[b] Mean percents followed by the same letter are not significantly different ($P < 0.05$; Duncan, 1955).

subflexa were caught in sticky traps containing the 5CB plus Z11–16:OH than in traps baited with blends that did not contain Z11–16:OH. The addition of Z7–16:Ac and Z9–11:OH to the five component blend had no significant effect on trap captures at the 500-μg load. Because Z7–16:Ac was found to be a component emitted by calling virgin female *H. subflexa*, it was included in all subsequent tests. None of the pheromone blends tested in this experiment, which were loaded with a total of 500 μg of material, caught as many males as traps baited with two virgin females. In experiment 2, analyses of trap capture of male *H. subflexa* obtained with septa loaded with a total of 1 mg of a 6CB plus Z11–16:OH indicated that Z11–16:OH was a necessary component in the pheromone blend (Table 3). When Z11–16:OH was loaded with the 6CB (total of 1 mg), trap captures were not statistically different from those obtained with traps baited with two virgin female *H. subflexa*.

The addition of Z9–16:OH to the 6CB resulted in low trap capture. Similar to experiment 1, the addition of Z9–16:OH to the 6CB plus Z11–16:OH provided no increase in trap capture of males (Table 3). The addition of three other minor components previously reported as being present in extracts of the pheromone glands of females (Klun et al., 1982) resulted in no significant increase in trap capture. It was noted that the Klun blend released 3% more pheromone than the other blends tested in this experiment.

The effect of the percentage of Z11–16:OH on capture of male *H. subflexa* was evaluated using septa loaded with the 6CB plus increasing amounts of the alcohol using both sticky traps and bucket traps. Analysis of the mean percentage of males captured in sticky traps indicated that the greatest percentage of moths was caught with blends that contained 1.4, 3.1, and 5.8% of Z11–16:OH and released the alcohol at 0.9, 1.6, and 3.5% of the total blend (Figure 1). The mean percentage of moths caught in sticky traps baited with lures that released 1.6% of the alcohol was significantly higher than for septa that released no alcohol or when the alcohol was released at 4.5% of the total blend. Results of a similar experiment using bucket traps indicated that the optimum range of release rate of Z11–16:OH was more critical when using bucket traps than when sticky traps were used. In this experiment, septa that released Z11–16:OH at 1.6% of the total blend resulted in significantly higher trap captures than septa that released 0, 3.5, and 4.5% of the alcohol (Figure 1).

The effect of septa load of the 6CB with the addition of 3.1% of Z11–16:OH (1.6% emitted) from rubber septa on male capture was investigated using bucket traps. Septa containing a total of 0.3, 1, 3, 6, or 10 mg of pheromone were used and provided a release rate of 105–1110 ng/hr. The results (Figure 2) showed a linear increase ($y = 3.93x + 10.62$; $r^2 = 0.89$) in capture of males as the septa load was increased to 6 mg of pheromone (590 ng/hr released).

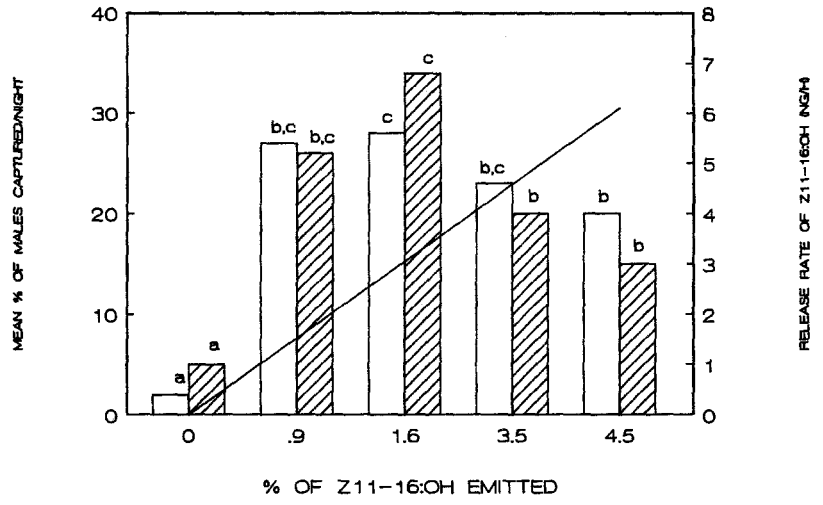

FIG. 1. Percent of male *H. subflexa* caught in sticky traps (open bars) and bucket traps (slashed bars) per night using six-component pheromone blend baited with increased percents of Z11-16:OH. Solid line is the release rate of alcohol (right y axis $y = 1.41x$, $r^2 = 0.95$). Open bars with the same letter are not significantly different and slashed bars with the same letter are not significantly different, Duncan's multiple range test ($P \leq 0.05$, $N = 15$).

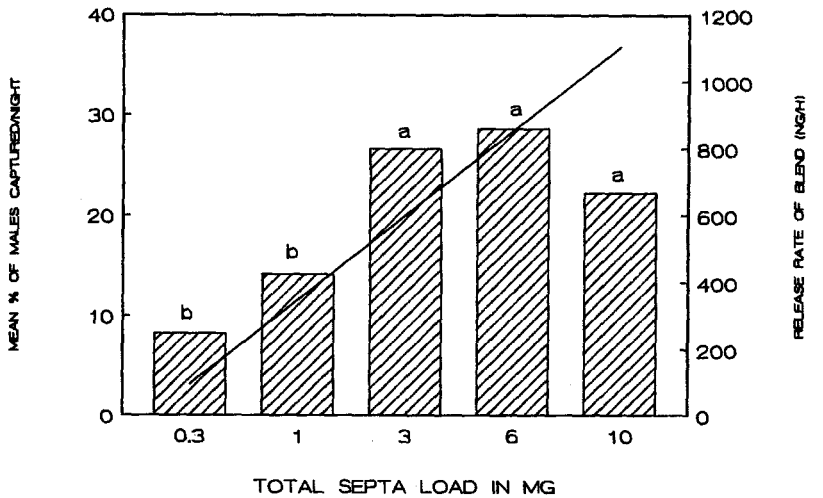

FIG. 2. Percent of male *H. subflexa* trapped per night in bucket traps baited with the seven-component pheromone blend at increased doses (bars). Bars with the same letter are not significantly different, Duncan's multiple range test ($P < 0.05$, $N = 17$). Solid line is the release rate of pheromone (right y axis $y = 104x + 65.4$, $r^2 = 0.92$).

DISCUSSION

Results obtained in this investigation indicate that Z11–16:OH is a critical pheromone component necessary for trapping male *H. subflexa*. Additionally, the release ratio of the alcohol in the seven-component blend required for optimum capture in sticky traps is in a narrow range: 0.9–3.5%. The release ratio range of the alcohol for maximum capture of males was reduced further when bucket traps were used. The importance of release rate of pheromone when used with bucket traps for capture of insects has been documented previously (Mitchell and Heath, 1986).

This study supports and further defines the results previously published on the pheromone system of *H. subflexa*. In the report by Teal et al. (1981), the addition of both Z9–16:OH and Z11–16:OH to a six-component blend resulted in decreased trap captures. The reported load ratios of the alcohols were 14.4% for the Z9–16:OH and 22.2% for the Z11–16:OH. Our results indicate that trap capture is reduced when the percentage release ratio exceeds 6%. Although release ratio data were not provided by Teal et al. (1981), it can be hypothesized that the release rate of the alcohols from the polyethylene vials used exceeded 6%. Using an 11-component synthetic blend containing 5.2% Z11–16:OH on cotton wicks, Klun et al. (1982) captured more male *H. subflexa* in traps baited with synthetic pheromone than in traps baited with three virgin females. Although no release ratio data were provided, it is probable that the percentage release of alcohol in the Klun blend was not sufficiently high to cause a reduction in trap catches.

The importance of Z11–16:OH as a pheromone component in members of the *Heliothis* genus appears to be related to species specificity. Raina et al. (1986) reported that this compound was critical for the attraction of male *H. phloxiphaga* (Grote and Robinson). Information regarding the release rate and ratio of the pheromone emitted from filter paper and cotton wicks used by Raina et al. (1986) was not provided. Thus, it is not known how precisely the blend must be formulated for optimum response of *H. phloxiphaga*. Teal et al. (1984) demonstrated that a low percentage of Z11–16:OH decreased trap capture of *Heliothis zea* (Boddie) when formulated in hollow fibers. Reports regarding the importance of Z11–16:OH in the attraction of *H. virescens* are variable (Sparks et al., 1979; Vetter and Baker, 1983; Ramaswamy et al., 1985). Resolution of the importance of this alcohol for attraction of *Heliothis* species may require, as was demonstrated in the present study for *H. subflexa*, formulations that provide release ratios and release rates of the pheromone blend with a high degree of precision.

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CHEMOSENSORY RESPONSES OF COWPEA WEEVIL, *Callosobruchus chinensis* TO AN AQUATIC WEED, WATER HYACINTH, *Eichhornia crassipes* (MART) SOLMS

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Abstract—The behavioral responses of cowpea weevil, *Callosobruchus chinensis* to the leaf extracts of the aquatic weed, water hyacinth, *Eichhornia crassipes* (Mart) Solms. was evaluated by three different methods. The petroleum ether-soluble fraction of *E. crassipes* induced high weevil attraction in all methods employed. The olfactometer assay provided a reading on weevil chemosensory responses, whereas the free-choice tests measured response and preference of weevils to the untreated or extract-treated food grain of their choice over a longer period. The extracts were also presented under no-choice test conditions to assay them for the presence of oviposition stimulants. In all these methods the extracts of water hyacinth evoked a quick directional response. Another interesting feature is that the extract, besides producing attraction, also caused mortality of the insects.

Key Words—Behavioral responses, cowpea weevil, *Callosobruchus chinensis*, Coleoptera, Curculionidae, water hyacinth, *Eichhornia crassipes*, olfactometer.

INTRODUCTION

Although chemosensory reactions have long been recognized as essential perceptive processes in Coleoptera associated with stored grain, only recently have a few studies examined this aspect of stored-product insects (Gomez et al., 1983). While attempting to evaluate the allomonic principles of *Parthenium hysterophorus*, Sharma and Toshi (1977) found that the acetone and methanol extracts of this weed were attractive to the red flour beetle, *Triboleum casta-*

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neum. Olfactory responses of rice weevil, *Sitophilus oryzae* to fractions of rice grains, and *Trogoderma granarium* to odors of wheat, feces, and fecal components were also reported (Honda et al., 1969; Stanic and Shulov, 1969). However, reports on the plant products affecting the chemosensory behavior of stored product pests are very scanty. In particular, the weeds and wild plants that are of no direct use for humans can be exploited for the protection of stored commodities from pest insects. A few such plants already have been reported to be useful for this purpose. The insecticidal value of the essential oil of *Acorus calamus* has been examined against the pulse beetle, *Bruchus chinensis* (Yadava, 1971). The oil protected the grains for many months from the attack of beetles. The efficacy of two wild plants, *Hyptis spicigera* and *Cassia nigricans*, in controlling the bean weevil, *Acanthoscelides obtectus*, was determined by Lambert et al. in 1985.

Water hyacinth, *Eichhornia crassipes* (Mart) Solms. is a floating aquatic weed of South American origin that has become a major problem in India. Dense mats of water hyacinth frequently pose problems in the commercial and recreational use of water, increase damage resulting from flooding, and cause major changes in the ecology of infested areas. Various control measures and utilization of this weed have been investigated extensively (Gopal and Sharma, 1981). Insecticidal and growth-regulatory properties of water hyacinth against a few storage pests are already reported (Rani and Jamil, 1989). In this investigation behavioral assay of chemical extracts of water hyacinth was undertaken to establish the presence of attractant and/or repellent properties that affect the behavior of the cowpea weevil. The bruchid beetles have obvious economic importance, because they breed on legumes and consume valuable proteins that would otherwise be eaten by man, reduce seed quality, and lower seed germination.

METHODS AND MATERIALS

Water hyacinth plants with long petioles were collected from a local pond near the laboratory. Leaves of the plants were separated and air-dried for three days. The dried leaves were coarsely ground by using a mortar and pestle. The ground material (500 g) was extracted with petroleum ether (bp 40–60°C) in a Soxhlet apparatus. The resulting extract was concentrated under reduced pressure to remove the solvent.

A stock solution containing 100 mg/ml of the extract in acetone was prepared and refrigerated when not in use. Lower concentrations of 80, 60, 40, and 20 mg/ml were obtained by dilution of the stock solution with the solvent. Only these dosages were used in all the departments.

Collosobruchus chinensis adults were obtained from laboratory cultures

maintained on green grain, *Vigna radiata*, which also were used in all the experiments described below. The grains were cleaned and later disinfested by keeping at 60°C for a 3-hr period. These samples were brought to equilibrium prior to use for experimental purpose. All cultures and experiments were conducted in a controlled temperature and humidity (CTH) room at 27 ± 1°C and 65–75% relative humidity using culturing procedures previously described by Dick and Credland (1984).

Beetles used in the tests were separated daily from the green grain culture media. Newly emerged weevils were selected because weevils younger than 4 days of age were more sensitive to odors than older ones (Honda et al., 1969). Also, possible sex pheromone effects would be minimized. According to Sharma & Deora (1980), sex pheromone may be produced and released by weevils 5 days old. The absence of pheromone effects was confirmed by two tests: (1) when 25 newly emerged females were caged in the sample tube, no attractant response was elicited from the unsexed adults in the stem of the Y tube; and (2) when 25 unsexed adults were caged in the sample tube, no aggregation response was elicited from the unsexed adults in the stem. These tests were replicated five times.

Olfactometer Assay. Attractancy and/or repellency of the extract for *C. chinensis* was assayed by studying the behavior and response of the weevils exposed to the compound. The apparatus used to study the olfactory behavior of the weevils is a Y-type olfactometer. The lengths of the stem and two arms of the Y tube are 20 cm and the diameter is 1 cm. Smaller tubes of 5 cm length and 1 cm diameter, which could fit snugly inside the ends of both the arms, were used for placing cotton swabs, each weighing 30 mg, impregnated with 1 ml of sample (test) or acetone solvent (control). The solvent was evaporated by air-drying. The test and control cotton swabs placed in the smaller tubes were inserted at the open ends of the bigger tube. Twenty 1-day-old unsexed adult beetles were introduced into the olfactometer through the free end of the stem of the Y tube after a priming period of 30 min, during which the odor concentration built inside the two arms.

The distribution of 20 experimental insects in the control (odor-free) and test (odor-containing) sectors of the tube was recorded every 10 min for 2 hr. At least five such experiments were conducted, and the number of insects aggregating in the test and control sectors was averaged for all readings. Attraction and/or repulsion was determined by using the formula $[T - C/T + C] \times 100$ where T is the number of insects in the test and C in the control sectors. Index of attractancy above 50 indicated attraction; below 50 indicated repulsion.

Free-Choice Tests. To measure the preference of the weevils for treated and untreated areas, free-choice tests were conducted. The olfactometer was of the alternative chambers type and featured a glass trough (15 cm diam.). Three small petri plates (5 cm diam.) were cemented equidistantly around the wall,

and in each was mixed 32.5 g of green grain with various doses of the extract, 80, 60, 40, and 20 mg dissolved in 2 ml of acetone separately and after the solvent evaporated under an airstream. Grains treated with acetone alone served as controls. Twenty 1-day-old unsexed weevils were introduced in the center of the apparatus which was then closed with a thick cloth and elastic band. The experimental set up was kept in a temperature-controlled incubator (28 ± 1°C) that also provided darkness. The number of weevils found in each Petri plate was recorded 15, 30, and 60 min after introduction.

No-Choice Test. An experiment was conducted to test the effects of prolonged exposure to water hyacinth extract on *C. chinensis.* In this treatment, 20 weevils were placed individually into 50-ml plastic cups. The cups were filled with 10 g of grain sprayed and air-dried with 2 ml of the extract, at various concentrations. The cups were then capped with a thick cloth held in position with a rubber band. Twenty additional weevils placed in identical cups containing the grains with 20 ml of acetone, served as a control. Both treatments were held at 28 ± 1°C for 24 hr to compare adult mortality. For each dose, five replicates were used.

RESULTS AND DISCUSSIONS

The petroleum ether extract of powdered dry leaves (500 g) of water hyacinth yielded 4.72 g of a dark green thick liquid. This extract was reported to be toxic to cowpea weevils in contact as well as in food grain treatment methods (Rani and Jamil, 1989). In the present investigation, three types of behaviorial responses were tested. The olfactometer assay provided a reading on weevil chemosensory response to extracts during a 3-hr period. The free-choice tests measured response effects by permitting weevils to be attracted to or feed on extract-treated food grains of their choice over a longer period. Lastly, these same extracts were presented under no-choice test conditions to assay them for the presence of oviposition stimulants.

The petroleum ether-soluble fraction of *E. crassipes* induced high weevil attraction in most of the methods employed. In the olfactometer assay, the extract showed a high range of attraction to pulse beetles. Fifteen minutes after release of the weevil, the attraction index was significantly higher than the index value of the control. The response was more clear-cut 30 and 45 min after release. Within 10 min of their introduction into the olfactometer, insects started moving toward the treated arm. All aggregated on the cotton swab. After a period of 45 min, the insects died on the extract-impregnated cotton (Table 1). The results obtained at all concentrations tested were similar.

When weevils were allowed to crawl on grains containing extract, there

TABLE 1. RESPONSE OF PULSE BEETLES TO PETROLEUM ETHER EXTRACT OF WATER HYACINTH LEAF IN OLFACTOMETER

Dosage	Time (min)	Number of insects found on[a]		Index of attractancy	Mortality (%)
		Treated sector	Control sector		
80	10	1.0	0	100.0	Nil
	15	8.0	2.8	48.20	Nil
	30	13.2	2.4	69.20	10.0
	45	20.0	0	100.00	100.0
60	10	2.0	0	100.00	Nil
	15	8.0	2.5	52.40	Nil
	30	10.8	2.2	66.20	68.0
	45	20.0	0	100.00	100.0
40	10	1.0	0	100.00	Nil
	15	10.0	3.0	53.80	Nil
	30	15.2	2.1	75.70	Nil
	45	19.3	0	100.00	Nil
20	10	1.2	0	100.00	Nil
	15	4.0	3.2	11.11	Nil
	30	12.0	3.2	57.90	Nil
	45	17.4	1.8	81.20	Nil

[a] Mean of five replicates (20 insects per replicate).

was a well-defined reaction. The weevils were attracted in large numbers by treated grain and after a period of 1 hr all of them were found dead. This occurred in all the concentrations tested except the lower dilution, i.e., 20 mg/2 ml; at this dilution insects did not die but became sluggish.

In no-choice tests, the extract produced 100% mortality of the weevils at 80 and 60 mg/2 ml, and a lesser dose of 40 mg/2 ml caused 40% mortality of the insects within 24 hr. However, remaining insects were also found dead after a period of 72 hr. No lethal effect was noted at the dose of 20 mg/2 ml even after 72 hr of treatment (Table 2).

The leaf extract of water hyacinth contained compounds that evoked a quick directional response and also an insecticidal response. It is possible that the extract contains more than one chemical, one that serves as a stimulant on both sexes, and another that serves as a toxicant.

Further elucidation of the questions concerning the identity of the compound(s) involved in the behavioral responses of the rice weevil awaits further fractionation of the petroleum ether extracts and characterization of these frac-

TABLE 2. RESPONSES OF PULSE BEETLE TO WATER HYACINTH EXTRACT IN NO-CHOICE TEST

Dosage (μg/ml)	Mortality (%) after 24 hr	Mortality (%) after 72 hr
80	100	100
60	100	100
40	40	100
20	0	0

tions chemically and behaviorally. Goswami et al. (1983) reported a significant amount of stigmasterol (0.07% dry weight) in water hyacinth plants and the possibility of its enrichment 10-fold by anaerobic digestion of the plant material. Most of the sterols were present in the free form, which is significant in considering water hyacinth as a source of sterol. These findings were further confirmed by Jamil et al. (1988). Preliminary chemical analysis of the extract that caused molting abnormalities in *Corcyra cephalonica* revealed the presence of sterols.

The combined contact toxicity and attraction reported here make *E. crassipes* a good candidate for further development as a short-term grain protectant against infestation by cowpea weevils.

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SEX ATTRACTION IN PAPER WASP, *Polistes exclamans* VIERECK (HYMENOPTERA: VESPIDAE), IN A WIND TUNNEL

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Abstract—A wind-tunnel bioassay was developed to test for the presence of sex attractants in the paper wasp, *Polistes exclamans* Viereck (Hymenoptera: Vespidae). Males exhibited significant upwind flight and attraction (chemoanemotaxis) in response to airflow passed over unmated females, and to hexane extracts of whole bodies and thoraces of unmated females. Unmated females were attracted to hexane extracts of males and to hexane extracts of each body tagma of males, suggesting distribution of the pheromone over the cuticle by grooming. The ectal mandibular and seventh sternal glands are the likely sources of the male-produced sex attractant(s) since extracts of each elicited female attraction in the flight tunnel. These glands are associated with gastral and mandibular rubbing of perch sites by territorial males of *Polistes* species.

Key Words—Insecta, mating, pheromone, attractant, Hymenoptera, Vespidae, *Polistes exclamans*, paper wasp.

INTRODUCTION

The importance of pheromones in mate-finding has been well studied in many insect species, most notably in pestiferous moths. However, the role of sex attractants in the mating biology of social wasps (Vespidae) is not well known. Various researchers have demonstrated experimentally close-range attractants and copulatory stimulants in paper wasps (Post and Jeanne, 1983a, 1984a), polybiines (Keeping et al., 1986), and hornets (Ono and Sasaki, 1987), and others have suggested close-range attractants in yellowjackets (*Vespula*) and hornets (*Vespa*) based on behavioral evidence (Akre, 1982; Batra, 1980; Ross,

1983). Few workers, however (e.g., Ono et al., 1985), have investigated the possibility that vespid pheromones attract mates over long distances. We investigated the role of chemoanemotactic responses to paper wasp pheromones, and thus long-range attraction, in the formation of dispersed mating swarms of sexuals of the paper wasp, *Polistes exclamans* Viereck.

Polistes wasps typically mate away from the nest (Noonan, 1978). In the fall (September–November) males of several temperate species defend and scent-mark perches, presumably for mating purposes (Lin, 1972; Post and Jeanne, 1983b; Turillazzi and Cervo, 1982; Wenzel, 1987) and often form aggregations on hilltops or other prominent landmarks (Beani and Turillazzi, 1988; Mathes-Sears and Alcock, 1986). Males typically press the last few gastral sternites against their perch (Post and Jeanne, 1983b), and some species also rub their mandibles on the substrate (Wenzel, 1987). These behaviors, in conjunction with the presence of large mandibular and sternal glands, strongly suggest that males release pheromone at such sites in order to attract potential mates. Females are known to visit these male perch sites within these aggregations, although infrequently (Mathes-Seares and Alcock, 1986). However, to date, no experimental evidence shows that females are attracted to such marked sites or to male aggregations. In contrast, female paper wasps do not exhibit any obvious calling posture or scent-marking behavior, but Post and Jeanne (1984a) demonstrated that they possess an interspecific, close-range sex pheromone in the venom and a species-specific surface pheromone on the thoracic and gastral cuticle.

We present here demonstrations of attraction in a wind-tunnel bioassay by *Polistes exclamans* males to the airflow passed over unmated females and to extracts of unmated females and, similarly, by unmated females to extracts of males.

METHODS AND MATERIALS

Greenhouse Colonies. Polistes exclamans colonies were collected during late summer and early fall (August–mid-October) in Gainesville, Florida. Adults were vacuumed off nests into Plexiglas traps (Akre et al., 1973), and nest combs were then recovered. Adults were refrigerated until incapable of flight. Males and females were then placed into separate cages (56 × 56 × 57 cm, 28 × 29 × 30 cm, or 21 × 21 × 21 cm) and maintained in different rooms of a greenhouse. Combs were placed in cages and examined daily. Males and females were removed and placed in their respective cages. Individuals from different colonies were placed into separate cages but were mixed after mating trials (see below). Honey and water were placed in each cage. Most caged wasps actively

flew between 11 AM and 5 PM (EDT) but aggregated in a corner of their cage the rest of the day. This activity period is similar to the field behavior observed in Florida. Warm temperatures (23–32°C) and bright sunshine (1000–2500 ftc) were required for optimum flight activity. Consequently, wind-tunnel bioassays were done on warm and mostly sunny days during the peak activity periods of the wasps (11 AM to 3 PM).

Females collected on nests should be uninseminated since mating probably occurs away from the nest (Noonan, 1978). In this study, 63 females from collected colonies were dissected and examined for sperm at different times during the fall. Forty-two females sampled from late August to late October were unmated, while five of 21 females sampled in November had sperm in their spermathecae. Thus, larger, fat-laden females from colonies collected from late August through mid-October were likely to be unmated gynes (see Strassman, 1984, for criteria distinguishing workers and gynes) and were selected for use in the subsequent mating trials.

Mating trials were conducted October 10, 13, and 19, 1988, using males and females selected randomly from different colonies to obtain a large pool of females and males showing some sexual activity. One male and one female were placed in a cylindrical plastic container (1 liter) and observed for 5 min. The number of antennations and mounting attempts were recorded for the male, and antennations, escape attempts, and bouts of biting or chasing the male were recorded for the female. Only a few females were successfully mated (four of 324 couplings), because most females were unreceptive to males. A low level of female receptivity was also observed in mating trials with *Polistes fuscatus* (Larch and Gamboa, 1981; Post and Jeanne, 1983c). Approximately half the females (166) elicited two or more mounting attempts by males. These "attractive" unmated females and their "aggressive" male suitors were then randomly used in the wind-tunnel bioassay and as a source of extracts. These male suitors were housed in two large (56 × 56 × 57 cm) cages each containing ca. 80 wasps. The females used were held in two large cages in a room separate from the males.

Wind-Tunnel Bioassay. All bioassays were conducted in a greenhouse in a Plexiglas flight tunnel (0.6 × 0.6 × 2.2 m) positioned 35 cm in front of an exit fan, thereby creating a pulling-type wind tunnel (Baker and Linn, 1984). Seven layers of plastic screen were applied to the upwind end of the tunnel to reduce airspeed to 1 mph (~28 m/min) and create a more laminar airflow. The point source of the volatiles (cotton wick or brass nozzle) was positioned at the upwind end of the tunnel, 16 cm below the top and 10 cm from the upwind end. The plume geometry was determined by releasing titanium tetrachloride at this height (16 cm) from the upwind end and marking the limits of the plume

on the sides of the tunnel. This plume height was below the typical flight level of the wasps in the wind tunnel, as they generally flew up against the top or the upper 1/6 of the tunnel. Wasps were released 1.8 m downwind from the source in the center of the plume from a screen cage (7 cm diam., 7.5 cm height). The release platform was a 15-cm-diam. watch glass resting on a ring stand 30 cm below the top of the tunnel. Test insects were placed in the release cages 1–2 hr before the bioassay to minimize handling immediately prior to bioassays. The release cage with a single test insect was gently placed on the watch glass at a slight incline with the opening facing downwind. This allowed the wasp to remain in the plume momentarily before it exited from the cage. Individual test wasps were observed for 2 min and scored for upwind flight, attraction, or chemoanemotaxis (zigzagging upwind flight within the plume; Kennedy, 1983), close-range casting in front of the source (= hovering), and contact with the source. The length of oriented upwind flight from start to closest approach was also noted. Wasps exhibiting attraction were not scored if only observed within 20 cm of the source since this may occur as a visual response (Post and Jeanne, 1984b). The percent responses were analyzed by chi-square test or were transformed (arcsin) and analyzed using ANOVA and Duncan's multiple-range test (Steel and Torrie, 1960). Light intensity, temperature, and relative humidity were recorded during all bioassays. One or two sets of bioassays were usually performed on each day. After each bioassay, tested wasps were returned to one of two large cages each housing ca. 80 wasps of the same sex. The same individuals were not tested on the same day, but were part of a pool of wasps from which selections were made for bioassays on subsequent days. This experimental design and protocol was followed in all subsequent bioassays.

Airflow Chamber Test. This experiment was designed to test the attractiveness of males to pheromones emanating from live females. A system was developed to introduce volatile chemicals from the females directly into the wind tunnel. Twenty unmated females were placed in a glass jar (3.8 liter) positioned on top of the flight tunnel at the upwind end. The jar was blocked from the view of males in the flight tunnel. The jar lid had inlet and outlet nozzles by which air was passed through the jar via a standard aquarium pump. The jar lid was sealed with parafilm to avoid leakage. The air from this chamber was conveyed into the wind tunnel with Tygon tubing and a vertical steel pipe. A brass elbow (i.e., nozzle) on the end of the steel pipe vented the female airstream into the tunnel. Airflow from the nozzle was regulated to 1 liter/hr using a flowmeter (Brooks Sho-Rate 1355). A system control consisted of an empty jar with an identical set up. Ten males were individually tested to the airflow from the empty jar, followed by 10 males flown individually to the airflow from the jar with 20 females. Seven trials of this experiment (N = 70

per sample) were conducted during October 11–18, 1988. The same jar was used for the control throughout the experiment. After each trial the jars and metal tubing and nozzle were washed in water, acetone, and hexane and left in sunlight to air dry. Outlet Tygon tubing was discarded after each trial.

Female Extract Tests. Male flight responses to hexane extracts of whole virgin females and body tagmata were performed in an attempt to isolate the source of a female-produced attractant. Samples of 10 freshly freeze-killed female whole bodies, heads, thoraces, and gasters were each ground with a mortar and pestle in hexane. Each sample was reduced to 2 ml [1 female equivalent (FE)/200 μl] under a N_2 stream and kept in the freezer until tested. The four extract samples were each tested in the same trial at a dose of 1 FE/200 μl hexane in the following sequence: blank (200 μl hexane), head, thorax, gaster, and whole body. Four males were flown to each sample during one trial (20 males per trial), and 10 trials were performed (40 males/sample) during November 3–14, 1988. The extract or solvent was poured onto a brown cotton wick (dyed with Rit cocoa-brown 20 fabric dye) positioned at the upwind end of the tunnel. The wick was mounted on a pin glued to a wire suspended from the tunnel ceiling. The solvent was allowed to dry for 1 min before commencing the bioassay. The wick was removed after each trial and the pin rinsed in hexane and allowed to dry for 5 min while air was flowing through the tunnel.

Male Extract Tests. Responses of virgin females to male extracts were also bioassayed in this wind tunnel in two separate experiments. The first experiment involved females flown to hexane extracts of freshly freeze-killed male whole body, head, thorax, and gaster. These four extracts were prepared in the same manner as in the female extract tests and were tested at a dose of 1 male equivalent (ME)/200 μl. The samples were tested sequentially: blank (200 μl hexane), head, thorax, gaster, and whole body. Four females were flown to each sample during one trial (20 females per trial), and 10 trials were performed (40 females/sample) during November 17–December 3, 1988. The bioassay protocol was the same as described for the female extract tests.

The second experiment was designed to determine more specifically the source of a male-produced attractant. The seventh sternum with its associated gland from 10 freshly freeze-killed males was dissected, placed in 2 ml of hexane, and allowed to soak for 24 hr in a freezer. An aliquot was removed and kept in the freezer until tested. Mandibles and their associated glands from 10 freshly freeze-killed males were dissected in saline and placed in 2 ml of hexane for 24 hr in the freezer. The ectal mandibular gland reservoir was broken with forceps. Extracts were tested at 1 male equivalent (200 μl). These two extracts, a control (200 μl hexane), and a standard (1 head equivalent/200 μl hexane) were tested in the following sequence: blank, sternal gland, mandibular glands,

and head. As before, four females were flown to each sample during a trial (16 wasps per trial), and the test was repeated 10 times (40 wasps per sample) during December 6–14, 1988.

RESULTS

Twenty-two of 70 *Polistes exclamans* males exhibited oriented upwind flight within the effluent plume, i.e., chemoanemotaxis, in response to an airstream passed over *P. exclamans* females, but none was attracted to the airflow from an empty jar (Table 1, A). In response to such female volatiles, males also exhibited significantly more upwind flight than in the control trials (Table 1, A). Close-range hovering downwind of the effluent vent occurred infrequently in both cases, and no source contacts were observed. Hovering in front of the nozzle may be a visual response since preliminary trials ($N = 56$, 3 males/trial) revealed no difference in hovering responses of a test (46%) and blank wick (39%) presented simultaneously in the wind tunnel ($\chi^2 = 1.47$, $P = 0.23$). In the field, males and females frequently inspect small objects projecting from a substrate.

Males also exhibited chemoanemotaxis to whole body extracts of unmated females (35%; $N = 40$) significantly more than to hexane alone (3%) (Table 1, B). Each female body tagma also elicited some attraction but only the thorax extract elicited a significantly higher response (25%) than the blank. Although

TABLE 1. PERCENTAGE OF FLIGHT RESPONSES OF MALE WASPS TO (A) AIRFLOW FROM 20 UNMATED FEMALES ($N = 70$ MALES) AND (B) FEMALE EXTRACTS (1 FE/200 μl HEXANE) ($N = 40$ MALES)[a]

Sample	Upwind flight	Chemoanemotaxis	Hover
A. Unmated females			
Empty jar	59a	0a	3a
Female jar	87b	31b	10a
B. Female extracts			
Blank	48a	3a	5a
Head	65ab	18ab	3a
Thorax	70b	25b	10a
Gaster	65ab	15a	3a
Whole body	93c	35b	3a

[a] Values with the same letters in the same column are not significantly different at $P = 0.05$ (A: χ^2 test; B: Duncan's multiple-range test on transformed data).

extracts stimulated more upwind flight than the blank, only the whole body and thorax extract values were statistically greater than the blank values (Table 1, B). As observed in the airflow chamber, test males infrequently hovered in front of the wick (3–10%, Table 1, B) and did not contact the source. Males attracted to the extracts or airflow did not usually fly the entire length of the tunnel ($\bar{X}$ = 68.8 ± 55.6 cm) but often deviated from their forward flight to fly sideways, downwind, or up against the ceiling of the tunnel.

Virgin female wasps exhibited behavior patterns similar to those observed in males, except that the former had a greater tendency to hover and contact the blank or test wick (Table 2). The length of female oriented upwind flight ($\bar{X}$ ± SD = 98 ± 58.5 cm; N = 116) was significantly greater than that of males ($\bar{X}$ ± SD = 68.8 ± 55.6 cm; N = 64) (t test, P = 0.001). Attracted females also reoriented to the plume in the 2-min period (42%, N = 101) more than did the males (8%, N = 59) (χ^2 = 18.11, $P = 2.1 \times 10^{-5}$).

Females were significantly attracted to male whole body, head, thorax, and gaster extracts (25–38%; Table 2, A) as compared to the blank wick (0%, Table 2, A). The male head extract elicited the highest attraction response, although not significantly higher than the other body parts, and stimulated significantly more females to hover in front of the source wick (35%) than the blank wick (13%) (Table 2, A). In a subsequent experiment testing specific gland extracts, unmated females exhibited significant attraction to extracts of the sternal gland (38%), mandibular gland (40%), and head (45%). However, there was no sig-

TABLE 2. PERCENTAGE OF FLIGHT RESPONSES OF UNMATED FEMALES (N = 40) TO EXTRACTS OF (A) MALE BODY PARTS AND (B) MALE GLANDS (1 ME/200 μl HEXANE)[a]

Sample	Upwind flight	Chemoanemotaxis	Hover	Contact
A. Male body parts				
Blank	50a	0a	13a	0a
Head	83b	38b	35b	10a
Thorax	75b	33b	28ab	3a
Gaster	73b	25b	18ab	5a
Whole body	90b	35b	28ab	0a
B. Male glands				
Blank	70a	5a	23a	8a
7th sternal gland	88a	38b	28a	0a
Mandibular glands	80a	40b	38a	0a
Head	80a	45b	48a	8a

[a]Values with same letters are not significantly different at P = 0.05 (Duncan's multiple range test on transformed data).

nificant close range response (hover or contact) (Table 2, B). Although each extract stimulated more upwind flight than did the blank, the differences were not significant.

DISCUSSION

Previous research on paper wasp mating biology has documented different potential mating strategies and a diverse array of male behavior. Our results constitute the first experimental evidence of attraction (chemoanemotaxis) of social wasp sexuals to pheromones produced by the opposite sex. Unmated female gynes are attracted to sex pheromones from the mandibular and seventh sternal glands of males (Table 2, B) and produce a sex pheromone that attracts males (Table 1). These attraction responses functioned over a distance of at least 2 m in the flight tunnel. The demonstration of a male-produced sex attractant supports the hypothesis proposed by several researchers that male scent-marking at perch sites attracts unmated females (Landolt and Akre, 1979; Post and Jeanne, 1983b,d; Wenzel, 1987). In addition, we propose that both female and male pheromones play a role in the formation of dispersed swarms of paper wasp sexuals. Such swarms could result from the attraction of female gynes to marking males and the subsequent response of conspecific males to take advantage of optimum signaling locations and upon arrival compete with other signaling males.

As in other species of paper wasps, *P. exclamans* males appear to mark their perches in the field by pressing their gastral sterna against the substrate. In field observations of perching and marking *P. exclamans* males, one male repeatedly marked perches on either side of a nest without brood but laden with females. Others marked perches at cracks and openings in a building that may have been hibernacula entrances. Gaster dragging is a prominent behavior exhibited by perching or patrolling males of several *Polistes* species (Post and Jeanne, 1983b; Turillazzi and Cervo, 1982; Wenzel, 1987), and mandibular rubbing has also been observed in perching *Polistes major* males (Wenzel, 1987); both behaviors are thought to be scent-marking in *Polistes* species. Examined males of *Polistes exclamans* had ectal mandibular glands (genal area = 0.52 mm^2, frons area = 0.50 mm^2) with an outer cell layer surrounding a membranous sac similar to those described for other vespids [Spradbery (1973) for *Vespula*; Landolt and Akre (1979) and Wenzel (1987) for *Polistes*]. Males also had developed (with globose acini; Landolt and Akre, 1979) glands on the seventh abdominal sterna, with no glands evident on sterna 4–6 (Downing et al., 1985). An examination of males of other *Polistes* species (*P. annularis*, *P. dorsalis*, *P. bellicosus*, *P. fuscatus*, *P. metricus*) revealed ectal mandibular

glands similar to those in *P. exclamans* (unpublished data). The widespread existence of probable scent-marking behavior and both ectal mandibular and abdominal sternal glands in males of other *Polistes* species support our expectation that male-produced sex attractants occur in other species of *Polistes* as well.

Although male attraction to male-produced pheromone was not investigated in this study, it is possible that such responses occur in nature. Several *P. exclamans* males and a few *P. metricus* males were observed visiting perch sites of marking *P. exclamans* males. This may also account for the large heterospecific aggregations of *Polistes* males and females we have observed at tall buildings, trees, and hills in Florida. However, further research is needed to demonstrate intrasexual and heterospecific attraction to these male-produced pheromones.

The pheromone of the female *P. exclamans* attractive to males appeared to be present on the entire outside of the female wasp, since extracts of each tagma were active in a flight-tunnel bioassay. Highest response rates, however, were obtained with extracts of the thorax (Table 1, B), which contains a pair of prothoracic and mesothoracic glands (Landolt and Akre, 1979) that could be a source of an attractant pheromone. Secretions from this or other exocrine glands, such as the venom reservoir or Dufour's gland, could conceivably be spread over the entire body during grooming. Keeping et al. (1986) and Post and Jeanne (1984a) showed that in other vespid wasps short-range chemical signals are present on the head, thorax, and gaster and also suggested grooming as a possible means of spreading the pheromone over the entire body. Each body tagma of the males elicited female attraction (Table 2), also suggesting distribution of the pheromone by grooming.

The flight-tunnel design and bioassay procedures used in this study should be useful in further research on sexual attraction in other wasp species and will facilitate the isolation and identification of sex pheromones evident here. Information on factors that affect pheromone release or attraction response (e.g., age, time of day, previous exposure, learning, etc.) can now be investigated using this bioassay to clarify the environmental context of this sex pheromone system. Field trials also will be necessary to form conclusions concerning the role of sex attraction in the mating strategies of these wasps.

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COMPOUNDS MODIFYING MALE RESPONSIVENESS TO MAIN FEMALE SEX PHEROMONE COMPONENT OF THE CURRANT BORER, *Synanthedon tipuliformis* CLERK (LEPIDOPTERA: SESIIDAE) UNDER FIELD CONDITIONS

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Abstract—Various blends of (*E*,*Z*)-2,13-octadecadien-1-ol acetate (*E*,*Z*2,13-18:OAc), (*Z*)-13-octadecen-1-ol acetate (*Z*13-18:OAc) (two previously identified pheromone components of the currant borer, *Synanthedon tipuliformis* females), (*E*,*Z*)-, (*Z*,*Z*)-3,13-octadecadien-1-ol acetates (*E*,*Z*- and *Z*,*Z*3,13-18:OAc), and *Z*,*Z*-3,13-octadecadien-1-ol (*Z*,*Z*3,13-18:OH) were evaluated in field traps in three geographic regions. Male *S. tipuliformis* were attracted to *E*,*Z*2,13-18:OAc in Tasmania, New Zealand, and Hungary. Captures were not influenced by the addition of *Z*13-18:OAc, tested in Tasmania and Hungary. In Hungary and New Zealand, the addition of *E*,*Z*3,13-18:OAc to *E*,*Z*2,13-18:OAc in a ratio of 100:3 was strongly synergistic; however, in Tasmania captures were reduced. The addition of *Z*,*Z*3,13-18:OAc to *E*,*Z*2,13-18:OAc in a ratio of 10:1 resulted in strong inhibition of trap captures in Hungary. When *Z*,*Z*3,13-18:OAc was added in different ratios to a 100:3 binary mixture of *E*,*Z*2,13-18:OAc/*E*,*Z*3,13-18:OAc in

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Hungary, it strongly reduced captures at, or above a ratio of 100:3:3 but no decrease was recorded at a ratio of 100:3:1. In New Zealand and Tasmania it reduced captures at a ratio of 100:3:1. Observations of behavioral responses of male *S. tipuliformis* in Hungary to synthetic baits in the field showed that *E*,*Z*2,13–18:OAc by itself evoked close-range approaches to the source from only 20% of males, whereas the addition of *E*,*Z*3,13–18:OAc in a ratio of 100:3 raised that value to 65%. Landing on the source was significant only at sources with a 100:3:0.1:3:10 blend of *E*,*Z*2,13–18:OAc/*E*,*Z*3,13–18:OAc/*Z*,*Z*3,13–18:OAc/*Z*,*Z*3,13–18:OH/*Z*13–18:OAc. A 100:3 binary mixture of *E*,*Z*2,13–18:OAc/*E*,*Z*3,13–18:OAc in a dose range of 10–1000 μg can be recommended for more effective field monitoring of *S. tipuliformis* populations in Hungary and in New Zealand. In Tasmania, at present, *E*,*Z*2,13–18:OAc by itself is the most potent sex attractant of the species.

Key Words—Currant borer, *Synanthedon tipuliformis*, Lepidoptera, Sesiidae, (*E*,*Z*)-2,13-octadecadien-1-ol acetate, (*E*,*Z*)-3,13-octadecadien-1-ol acetate, (*Z*,*Z*)-3,13-octadecadien-1-ol acetate, sex pheromone, trapping synergist, trapping inhibitor, field trapping, sexual behavior, geographically isolated populations.

INTRODUCTION

Several years ago, Voerman et al. (1984) found that (*E*,*Z*)-2,13-octadecadien-1-ol acetate (*E*,*Z*2,13–18:OAc) attracted male currant borer (or currant clearwing) *Synanthedon tipuliformis* Clerk. (Lepidoptera, Sesiidae). Shortly thereafter, Szöcs et al. (1985) showed that this compound and minor amounts of (*Z*)-13-octadecen-1-ol acetate (Z13–18:OAc) were present in extracts of the pheromone gland of females. Z13–18:OAc was inactive by itself but, when added to *E*,*Z*2,13–18:OAc in different ratios, in general slightly increased the number of males captured. These differences were not always statistically significant or consistent (Szöcs et al., 1985).

Priesner et al. (1986) reported that *E*,*Z*3,13–18:OAc added to *E*,*Z*2,13–18:OAc in ratios of 100:3 and 100:10 caught more *S. tipuliformis* males than *E*,*Z*2,13–18:OAc alone, while at the ratio of 10:3 moths were trapped in lower numbers. Electrophysiological studies support the existence of three types of receptor cells on the antennae of male *S. tipuliformis*, responding to *E*,*Z*2,13–18:OAc and to (*E*,*Z*)-3,13-octadecadien-1-ol acetate (*E*,*Z*3,13–18:OAc) and (*Z*,*Z*)-3,13-octadecadien-1-ol acetate (*Z*,*Z*3,13–18:OAc), respectively (Voerman et al., 1984; Priesner et al., 1986). (*Z*,*Z*)-3,13-octadecadienol (*Z*,*Z*3,13–18:OH) also was found to evoke responses from male antennae in EAG measurements (Voerman, personal communication).

In Hungary, in 1984, we initiated a study of the activity of mixtures of the above compounds in trapping experiments and in behavioral field observations in order to improve monitoring of field populations of *S. tipuliformis*. The cur-

rant borer is widespread in Eurasia from where it has been introduced to other continents, including Australia and New Zealand (Issekutz, 1955). Since differences may have evolved between these populations, comparison of the field performance of different attractant blends in these areas could be of importance, not only from a theoretical point of view, but also for practical usage. Trapping tests were conducted, therefore, in Tasmania (Australia) and New Zealand as well as in Hungary.

METHODS AND MATERIALS

Chemicals. *E,Z*2,13–18:OAc was prepared as described previously (Schwarz et al., 1983). The synthesis of *Z*13–18:OAc has been reported by Szöcs et al. (1985). *E,Z*3,13–18:OAc and *Z,Z*3,13–18:OAc were commercial products and *Z,Z*3,13–18:OH was obtained from the latter. All acetates were purified by high-pressure liquid chromatography on two stainless-steel columns (each 25 cm × 10 cm ID) that were plumbed in series and had been packed with 10 μm silica impregnated with 20% $AgNO_3$ using toluene as the eluent (4 ml/min) to give products that were both chemically and geometrically pure. All compounds were prepared and/or purified at the Insect Chemical Ecology Laboratory, Beltsville, Maryland.

Dispensers for Traps. Dispensers for traps were prepared at the Plant Protection Institute, Budapest, Hungary. The required amounts of compounds were applied in 10 μl hexane solvent to 1 × 1-cm pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ 9691/6; extracted three times in boiling 70% ethanol for 10 min, then also three times in methylene chloride for one day, prior to usage). The loaded dispensers were then individually wrapped in aluminum foil and stored at −60°C until use or sent by air mail to Australia and to New Zealand.

Trapping. A randomized complete block design was used in all tests. In Hungary, Tetra traps (Arn et al., 1979) (in 1985), or transparent plastic traps of similar shape and size (in 1986 and 1987) were used. Traps within a block were ca. 8–10 m (1985) or 3–4 m (1986 and 1987) apart from each other. The distance between blocks varied from 50 to 200 m. Traps were moved one position forward within a block twice weekly (1985 and 1986) or every day (1987). At the same time, captured males were recorded and sticky inserts of the traps changed. Traps were hung on currant shoots at a height of ca. 1 m above ground level. Tests were conducted in plantations of black currant (Kemence, Pest County) and red currant (Bernecebaráti, Pest County).

In Tasmania (1984–1985), plastic prism traps were used with 10-m spacings within blocks and 4-m spacings between blocks. Treatments were rotated as in Hungary. In 1986, because of space limitations in available currant plan-

tations, the within- and between-block spacings were 5 and 6 m, respectively. In 1987, these spacings were 3 and 10 m, respectively. For the site of the experiments, a black currant plantation at Slateford, Tasmania, was chosen.

In New Zealand, the within- and between-block spacings were ca. 10 m and 120 m, respectively. Traps (Zoecon ICP design) were rotated within a block, sticky trap inserts exchanged, and captures recorded twice a week (1985–1986) or each day (1986–1987). Trapping was conducted in a black currant plantation at Irwell, Canterbury, South Island.

In statistical analysis the capture of a trap recorded on a given date was regarded as a repetition. Capture data were transformed to log $(x + 1)$, and differences between mean catches were tested for significance by Duncan's new multiple-range test.

Behavioral Observations. For bait, various amounts of compounds were applied in 10-μl hexane solution to a 1-cm^2 piece of filter paper just before testing in the field. Filter papers were attached to ca. 5 × 1-cm thin plastic flaps. "Combined dead female" baits were prepared by attaching a dead *S. tipuliformis* female to the above-mentioned filter paper. The compounds then were applied to the filter paper in 10 μl of hexane.

The diel activity of the response of males was determined using the plastic traps baited with 10 μg of a 100:3 binary mixture of *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc. Ten traps were set out on July 1, 1987, at 2000 hr. On the following day, captures were first recorded (in each case sticky inserts exchanged for new ones) at 0800 hr, then hourly until 2000 hr.

Observational Methods. Field observations were conducted at the same sites as the trapping tests in Hungary. During behavioral observations, a freshly prepared bait was attached to the upper part of a currant shoot, ca. 1 m above the ground. In order to make arriving moths more visible, the bait was positioned centrally ca. 15 cm above a white sheet (30 × 20 cm). After testing, both the bait and the sheet were removed from the field and discarded.

Observations were made in sunshine and when the wind velocity was below ca. 5 m/sec, as we previously found that cloudy weather and wind hampered the flight of moths (Szöcs and Tóth, unpublished). Often males were attracted in such large numbers that they disturbed one another. In these cases, the observations were suspended and continued in places 80–100 m or further apart. The order of treatments tested was randomly changed in subsequent observations. The behavior of arriving males was described orally by the observer with a casette recorder.

The behaviors recorded and their measured parameters were defined as follows: (1) Approach: an orienting male flew up to ca. 2 cm distance from the bait. The number of males approaching the source was calculated as a percentage of the number of males appearing downwind from the bait at a distance of ca. 40 cm. Approach time is the time it took a male to fly from ca. 40 cm to

ca. 2 cm downwind of the bait. (2) Landing: following an approach the male alighted on the filter paper. The number of males landing was calculated as a percentage of the number of males appearing downwind from the bait at a distance of 40 cm.

RESULTS

Trapping

Effect of Z13–18:OAc on Trap Captures at E,Z2,13–18:OAc *in Tasmania and Hungary.* In trials in Tasmania and Hungary, different amounts of Z13–18:OAc were added to *E,Z*2,13–18:OAc. Although a rather wide range of ratios (1–100%) was evaluated, none of the blends tested caught significantly more males than the diene alone (Table 1). The monoene alone did not catch males.

In another trial in Hungary (June 11–July 1, 1985, Bernecebaráti, Pest County, five traps per treatments, checks were made twice a week), there again

TABLE 1. CATCHES OF MALE *S. tipuliformis* AT TRAPS BAITED WITH BLENDS OF *E,Z*2,13–18:OAc and Z13–18:OAc IN AUSTRALIA AND HUNGARY

Z13–18:OAc added (%)[a]	Mean No. moths/trap/check	
	Australia[b]	Hungary[c]
0	2.71 a[e]	1.74 ab
1	4.10 a	0.90 b
5	not tested	2.04 ab
7.5	not tested	1.10 b
10	2.73 a	2.90 a
20	3.23 a	2.08 ab
40	2.90 a	not tested
50	not tested	2.02 ab
100	not tested	1.00 b
300[d]	not tested	0.00 c
Unbaited	not tested	0.00 c

[a] In a percentage of *E,Z*3,13–18:Ac. The amount of *E,Z*2,13–18:OAc maintained at 100 μg (Australia) or 300 μg (Hungary).

[b] December 14, 1984–February 20, 1985, Slateford, Tasmania; No. of traps per treatment: 8; checks were made weekly.

[c] June 11–July 4, 1985, Kemence, Pest County; No. of traps per treatment: 10; checks were made twice weekly.

[d] μg, without *E,Z*2,13–18:OAc.

[e] Means followed by same letter within a column not significantly different at $P = 5\%$ (Duncan's new multiple-range test).

was no significant increase in the number of captured males within the respective dose levels when *E,Z*2,13–18:OAc alone and mixtures of *E,Z*2,13–18:OAc and *Z*13–18:OAc in a ratio of 5:1 were compared: 0.76 vs. 0.84 (10 μg); 2.52 vs. 2.40 (50 μg); 1.44 vs. 1.48 (100 μg); 6.68 vs. 8.40 (500 μg) mean number of males/trap/check, respectively. In an earlier test, this was the only ratio that gave better results than *E,Z*2,13–18:OAc alone (Szöcs et al., 1985).

Effect of Two Positional Isomers on Trap Catches at E,Z2,13–18:OAc *in Hungary.* The numbers of males trapped with the 300:5 blend of *E,Z*2,13–18:OAc and *Z,Z*3,13–18:OAc were not statistically different from the captures with *E,Z*2,13–18:OAc alone (Table 2). When the ratio of *Z,Z*3,13–18:OAc was increased to 10:1 virtually no males were captured.

The addition of *E,Z*3,13–18:OAc to *E,Z*2,13–18:OAc in a ratio of 300:5 and 10:1 increased the captures more than six and three times, respectively (Table 2). No males were captured in traps with single components of either *E,Z*- or *Z,Z*3,13–18:OAc.

Mixtures containing 0.1, 0.3, 3, and 10% of *E,Z*3,13–18:OAc in *E,Z*2,13–18:OAc caught significantly more males than *E,Z*2,13–18:OAc by itself (Table 3). The largest number of males was captured at the 100:3 *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc mixture. Captures were reduced when the ratio of *E,Z*3,13–18:OAc in the blend was increased to 10:3.

In a dosage test of the 100:3 *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc binary mixture, catches gradually increased at higher doses and appeared to level off above 300 μg (Figure 1).

In a further test, *E,Z*2,13–18:OAc by itself was compared to the 100:3 blend with *E,Z*3,13–18:OAc at different dose levels. The binary mixture caught

TABLE 2. EFFECT OF ADDITION OF *E,Z*- or *Z,Z*3,13–18:OAc and Z13–18:OAc to *E,Z*2,13–18:OAc ON TRAP CATCHES OF MALE *S. tipuliformis* IN HUNGARY[a]

–18:OAc (μg)			
*E,Z*2,13	*E,Z*3,13	*Z,Z*3,13	Mean No. moths/trap/check
300			2.87 c
300		5	2.40 c
300		30	0.07 d
300	5		19.13 a
300	30		10.00 b
		300	0.00 d
	300		0.00 d

[a]June 11–24, 1985, Kemence, Pest County, Hungary; No. of traps per treatment: 5; checks were made twice weekly. For significance see Table 1.

TABLE 3. EFFECT OF ADDITION OF DIFFERENT AMOUNTS OF *E,Z*3,13–18:OAc TO *E,Z*2,13–18:OAc ON CATCHES OF MALE *S. tipuliformis* IN HUNGARY[a]

Amount (μg)		
*E,Z*2,13–18:OAc	*E,Z*3,13–18:OAc	Mean No. moths/trap/check
100		7.24 c
100	0.1	10.94 b
100	0.3	11.72 b
100	1	7.38 c
100	3	19.48 a
100	10	11.56 b
100	30	2.46 d

[a]June 26–July 18, 1985, Kemence, Pest County, Hungary; No. of traps per treatment: 10; checks were made twice weekly. For significance see Table 1.

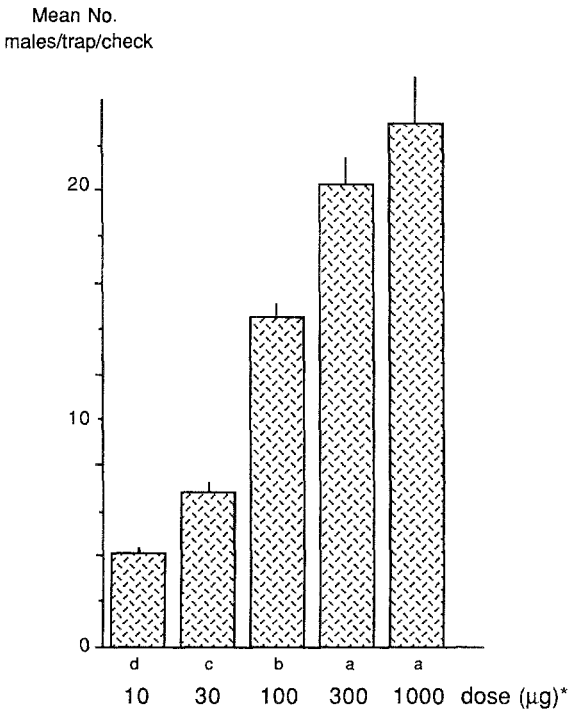


FIG. 1. Catches of male *S. tipuliformis* at different doses of a 100:3 mixture of *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc in Hungary. *Dose indicates the amount of *E,Z*2,13–18:OAc, in logarithmic scale. July 4–22, 1985, Bernecebaráti, Pest County, Hungary; No. of traps per treatment: 5; checks were made twice weekly. Columns with same letters are not significantly different (Duncan's new multiple-range test, $P = 5\%$). Bars represent SE.

significantly more males at the 100-, 10-, and 1-μg levels (ca. 6, 10, and 11 times, respectively) (Figure 2). No difference was found at 0.1 μg, where almost no males were trapped.

Effect of Z,Z3,13-18:OAc *on Trap Catches at 100:3 Blend of* E,Z2,13-18:OAc/E,Z3,13-18:OAc *in Hungary.* Z,Z3,13-18:OAc when added as a third component in ratios of 100:3:3, 100:3:10, or 100:3:30 to the binary *E,Z*2,13-18:OAc/*E,Z*3,13-18:OAc blend, dramatically reduced, or even eliminated captures (Table 4). When it was added in ratios of 100:3:1 and 100:3:0.1, no influence on captures was recorded.

Since, in several cases, pheromone inhibitors have been reported to act as synergists at extremely low percentages (Steck et al., 1982), in further trials Z,Z3,13-18:OAc was added as 0.1 part to the 100:3 mixture at different doses. At each dosage level the respective captures at the blend with Z,Z3,13-18:OAc were slightly higher (1.4-1.8 times increase), but these differences were not significant at $P = 5\%$ (Figure 3).

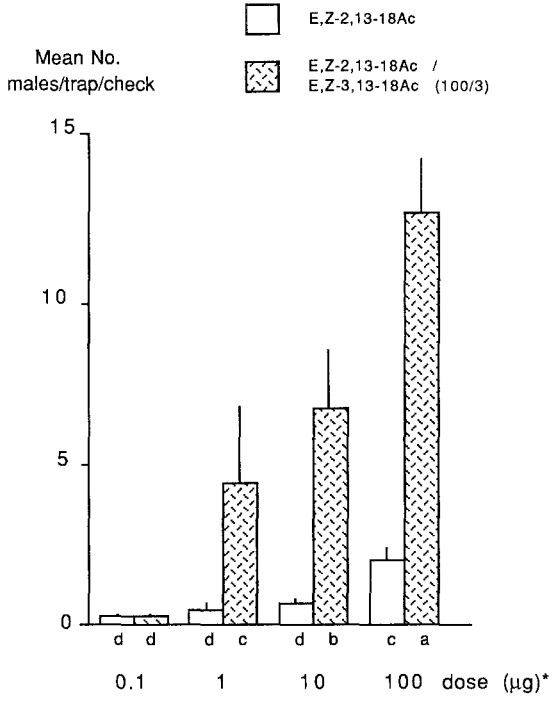


FIG. 2. Catches of male *S. tipuliformis* at traps baited with different doses of *E,Z*2,13-18:OAc alone, or with a 100:3 blend of *E,Z*2,13-18:OAc/*E,Z*3,13-18:OAc in Hungary. *Dose indicates the amount of *E,Z*2,13-18:OAc, in logarithmic scale. July 13-16, 1987, Kemence, Pest county, Hungary; No. of traps per treatment: 8; checks were made every day. For significance, see Figure 1.

TABLE 4. CATCHES OF MALE *S. tipuliformis* AT TRAPS BAITED WITH BLENDS COMPOSED OF 100:3 BINARY MIXTURE OF *E,Z*2,13–18:OAc WITH *E,Z*3,13–18:OAc AND *Z,Z*3,13–18:OAc IN HUNGARY[a]

*Z,Z*3,13–18:OAc added (μg)[a]	Mean No. moths/trap/check
0	10.69 a
0.01	11.65 a
0.1	11.25 a
0.3	1.88 b
1	0.38 c
3	0.00 c

[a]The amount of *E,Z*2,13–18:OAc maintained at 10 μg and that of *E,Z*3,13–18:OAc at 0.3 μg, respectively. July 13–15, 1987, Bernecebaráti, Pest County, Hungary; No. of traps per treatment: 8; checks were made every day. For significance see Table 1.

Comparison of Effect of E,*Z3,13–18:OAc on Catches at Traps Baited with* E,*Z2,13–18:OAc in Tasmania, New Zealand, and Hungary.* In the first trial, the addition of *E,Z*3,13–18:OAc to *E,Z*2,13–18:OAc in a ratio of 100:3 resulted in a statistically significant increase in captures in both New Zealand and Hungary (four and three times, respectively) (Table 5). In contrast, traps containing *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc in Tasmania caught males in significantly lower numbers than those baited with *E,Z*2,13–18:OAc by itself (Table 5). In a similar test in the subsequent season, although the capture of the 100:3 mixture did not differ significantly from that of *E,Z*2,13–18:OAc alone, the former was numerically lower (Table 6).

Comparison of Effect of Z,*Z3,13–18:OAc on Trap Catches at a 100:3 Blend of* E,*Z2,13–18:OAc/*E,*Z3,13–18:OAc in Tasmania, New Zealand, and Hungary.* Trap captures were reduced in Tasmania and New Zealand when *Z,Z*3,13–18:OAc was added as a third component to a mixture of *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc in a ratio of 100:3:1 (Table 5). In the Hungarian parallel trial the addition of *Z,Z*3,13–18:OAc in the same ratio did not influence captures.

The capture of the ternary mixture at a ratio of 100:3:0.1 did not significantly differ from that of the 100:3 binary mixture of *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc in Tasmania and Hungary. Such a difference was only recorded in the 1985–1986 trial in New Zealand (Tables 5 and 6).

At baits containing only 0.01% of *Z,Z*3,13–18:OAc, captures did not differ in any regions from those of the 100:3 binary mixture (Table 5).

Comparison of Effect of Z13–18:OAc and Z,*Z3,13–18:OH on Trap Catches at a 100:3 Binary Blend of* E,*Z2,13–18:OAc/*E,*Z3,13–18:OAc, and*

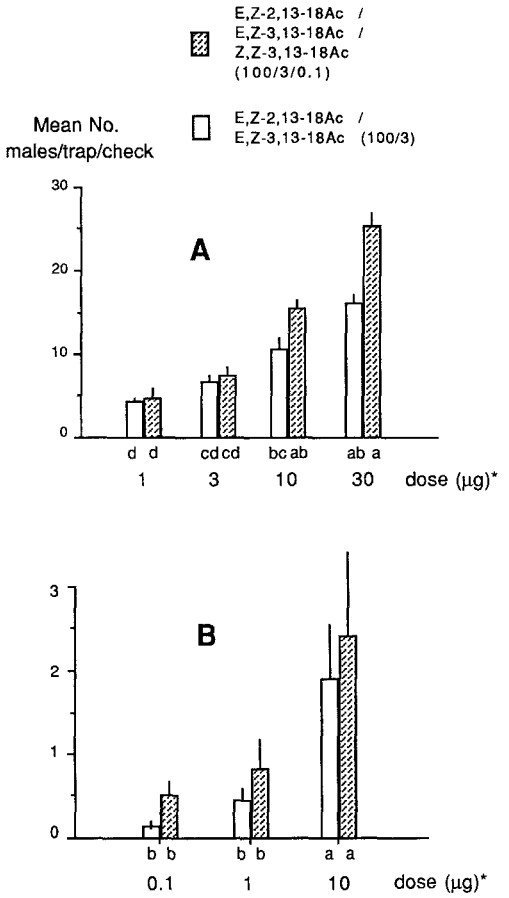


FIG. 3. Catches of male *S. tipuliformis* at different doses of a 100:3 and a 100:3:0.1 mixture of *E*,*Z*2,13–18:OAc/*E*,*Z*3,13–18:OAc and *E*,*Z*2,13–18:OAc/*E*,*Z*3,13–18:OAc/*Z*,*Z*3,13–18:OAc, respectively, in Hungary. *Dose indicates amount of *E*,*Z*2,13–18:OAc, in logarithmic scale. (A) July 7–11, 1986, Bernecebaráti, Pest county, Hungary; No. of traps per treatment: 8; checks were made every day. (B) July 14–25, 1986, Kemence and Bernecebaráti, Pest county, Hungary; No. of traps per treatment: 24; checks were made twice a week. Captures with same letters within a graph are not significantly different (Duncan's new multiple-range test, $P = 5\%$). Bars represent SE.

at a 100:3:0.1 Ternary Blend of E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc/* Z,Z*3,13–18:OAc in Tasmania, New Zealand, and Hungary.* For testing the effect of Z13–18:OAc and Z,Z3,13–18:OH on field performance of attractant blends, the 100:3 binary mixture of *E*,*Z*2,13–18:OAc/*E*,*Z*3,13–18:OAc and the 100:3:0.1 ternary mixture of *E*,*Z*2,13–18:OAc/*E*,*Z*3,13–18:OAc/

TABLE 5. CATCHES OF MALE *S. tipuliformis* AT VARIOUS COMBINATIONS OF *E,Z*2,13-18:OAc WITH *E,Z*- AND *Z,Z*3,13-18:OAc AND *Z*13-18:OAc IN THREE COUNTRIES

−18:OAc (μg)				Mean No. moths/trap/check[a]		
*E,Z*2,13	*E,Z*3,13	*Z,Z*3,13	*Z*13	HUN[b]	AUST[c]	NZ[d]
100				15.38 a	6.73 a	21.25 d
100	3			40.81 b	3.24 b	96.81 a
100	3	1		35.63 b	0.51 d	50.88 c
100	3	0.1		41.69 b	1.18 bcd	75.58 b
100	3	0.01		39.25 b	2.20 bc	83.60 a
100	3	0.1	3	39.19 b	2.00 bcd	92.56 a
100	3	0.1	10	41.56 b	0.98 cd	78.29 ab
Unbaited				0.56 c	0.00	9.25 e

[a] For significance see Table 1.
[b] June 20–25, 1986, Kemence, Pest County, Hungary; No. of traps per treatment: 8; checks were made twice weekly.
[c] January 15–March 23, 1986, Huonville, Tasmania; No. of traps per treatment: 5; checks were made weekly.
[d] December 13, 1985–January 3, 1986, Canterbury, New Zealand, No. of traps per treatment: 8; checks were made twice weekly.

TABLE 6. CATCHES OF MALE *S. tipuliformis* AT VARIOUS COMBINATIONS OF *E,Z*2,13-18:OAc WITH *E,Z*- and *Z,Z*3,13-18:OAc, *Z,Z*3,13-18:OH and *Z*13-18:OAc IN THREE COUNTRIES

Amount (μg)					Mean No. moths/trap/check[a]		
*E,Z*2,13-18:Ac	*E,Z*3,13-18:Ac	*Z,Z*3,13-18:Ac	*Z,Z*3,13-18:OH	*Z*13-18:Ac	AUST[b]	HUN[c]	NZ[d]
10					0.19 a	not tested	not tested
10	0.3				0.15 a	7.30 a	27.13 a
10	0.3	0.01			not tested	9.30 a	23.74 a
10	0.3		0.3		not tested	7.23 a	20.94 a
10	0.3			1	not tested	7.65 a	25.80 a
10	0.3	0.01		1	not tested	10.23 a	26.96 a
10	0.3		0.3	1	not tested	9.63 a	23.16 a
10	0.3	0.01	0.3		not tested	10.35 a	23.88 a
10	0.3	0.01	0.3	1	0.16 a	8.73 a	28.95 a
Unbaited					not tested	0.03 b	2.59 b

[a] For significance see Table 1.
[b] January 21–April 5, 1987, Slateford, Australia; No. of traps per treatment: 9; checks were made twice a week.
[c] June 29–July 3, 1987, Kemence, Pest County, Hungary; No. of traps per treatment: 10; checks were made every day.
[d] December 19, 1986–January 9, 1987, Canterbury, New Zealand; No. of traps per treatment: 10; checks were made every day.

*Z,Z*3,13–18:OAc were chosen as basic baits, as they proved to be the two most potent attractant blends in our previous tests.

Results presented in Tables 5 and 6 show that neither Z13–18:OAc nor *Z,Z*3,13–18:OH affected captures significantly when added singly or in combination at different ratios to the two basic baits.

Daily Pattern of Trap Captures of S. tipuliformis *Males*

The highest hourly capture was recorded at 1700 hr. Fifty percent of the total daily capture (209 specimens) was trapped between 1600 and 1800 hr (sunset was at 1945 hr).

Behavior of Males Attracted to Different Blends in the Field

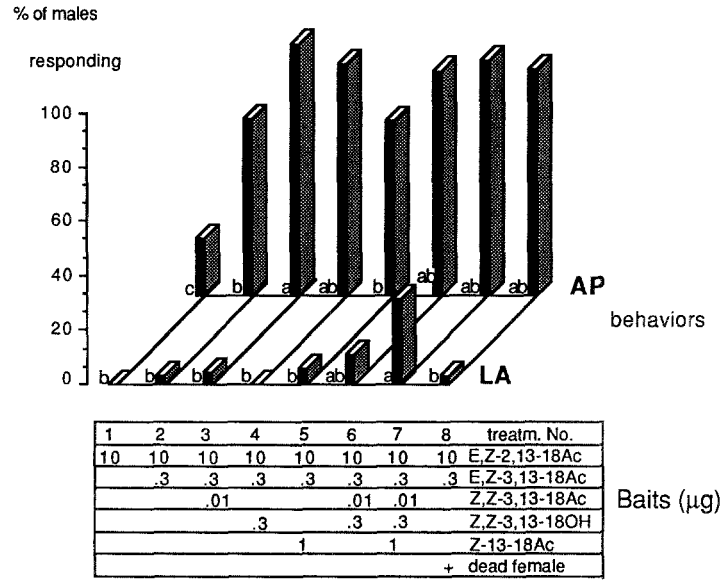
S. tipuliformis males were observed flying around the canopy of currant bushes on sunny afternoons during June and July. During their rapid zigzag flight, some males suddenly changed to a much slower straight upwind-directed flight; this was presumably in response to contact with the odor plume emitted from the pheromone bait. In our study, initiation of upwind flight was never observed at a distance greater than ca. 40 cm from the source. However, because the foliage obstructed observations beyond this distance, more precise estimates could not be obtained.

After starting upwind flight at ca. 40 cm downwind, males approached the bait to within ca. 2–3 cm and hovered there for some seconds. This was followed by landing on the pheromone source. Very often the behavioral sequence terminated in a close approach, i.e., hovering in front of the source. Males left the source by flying away. Sometimes they flew away to a distance of only 30–40 cm and, after a few seconds, flew back to the source, and, rarely, landed again. When approaching, the males extended their hairtufts at the end of the abdomen, and sometimes their claspers. In some cases they touched the source with their antennae while hovering. After landing, the males extended or continued to keep extended their hairtufts, and in several cases were observed to attempt copulation.

Responses to Different Synthetic Blends

Approach. *E,Z*2,13–18:OAc alone evoked approach from only 20.6% of males, a value significantly lower than those obtained at any other blend (Figure 4). At the 100:3 *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc mixture, a value of 65.6% was recorded. No significant difference was found between this binary mixture and any of the rest of the baits, with the exception of the ternary mixture containing *Z,Z*3,13–18:OAc. This latter blend gave the best response.

Landing. The highest value (31%) was recorded with the five-component



1	2	3	4	5	6	7	8	treatm. No.
10	10	10	10	10	10	10	10	E,Z-2,13-18Ac
	.3	.3	.3	.3	.3	.3	.3	E,Z-3,13-18Ac
		.01			.01	.01		Z,Z-3,13-18Ac
			.3		.3	.3		Z,Z-3,13-18OH
				1		1		Z-13-18Ac
							+	dead female

FIG. 4. Behavioral response of male *S. tipuliformis* to different attractant sources in the field. July 3–20, 1987, Kemence and Bernecebaráti, Pest County, Hungary. AP = approach; LA = landing. No. of males observed per treatment: 1 = 29, 2 = 32, 3 = 40, 4 = 14, 5 = 34, 6 = 35, 7 = 38, 8 = 31. Columns with same letters within a behavioral step are not significantly different (χ^2 test, $P = 5\%$).

blend, followed by a four-component blend (not containing *Z*13–18:OAc). The latter, however, did not elicit responses significantly different from the remaining blends. *E,Z*2,13–18:OAc on its own elicited no landings.

Approach Time. Mean approach time for males approaching sources containing *E,Z*2,13–18:OAc on its own was 4.33 sec, significantly longer than for any other blends (means range 2.16–2.59 sec, not significantly different from one another).

DISCUSSION

Field Trapping

The identification of *E,Z*2,13–18:OAc as a major pheromone component of *S. tipuliformis* was carried out with insects originating from Hungary (Szöcs et al., 1985). The field attractancy of this compound has been demonstrated only in Europe so far (Voerman et al., 1984; Szöcs et al., 1985; Priesner et al.,

1986). Field capture data presented in this paper show that males of both Tasmanian and New Zealand populations are attracted to this compound. The addition of the minor pheromone component, *Z*13–18:OAc, did not influence captures in Tasmania and Hungary.

The two positional isomers, *E,Z*- and *Z,Z*3,13–18:OAc, which had previously been reported to be active in single sensillum measurements (Voerman et al., 1984), exerted a remarkable influence on trap captures at *E,Z*2,13–18:OAc in our study.

*E,Z*3,13–18:OAc is a trapping synergist of *E,Z*3,13–18:Ac in ratios of 100:0.1–100:10 (with a maximum at 100:3) in Hungary. At a ratio of 10:3, however, it acts as an inhibitor. Its strong synergism in a ratio of 100:3 also was recorded in New Zealand, where it has successfully been used also in other studies for monitoring (Thomas and Burnip, 1989), while in Tasmania no synergism but rather a slight inhibitory effect was noted.

*Z,Z*3,13–18:OAc clearly inhibited captures in Hungary, when added to *E,Z*2,13–18:OAc in a ratio of 10:1. Similarly, when it was added as a third component to the mixture of *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc in a ratio of 100:3:3 (in Hungary) or of 100:3:1 (in New Zealand and in Tasmania), a significant decrease in captures was detected. No statistically significant increase could be achieved by a mixture of these three compounds in a ratio of 100:3:0.1, although in Hungary catches with such blends were always numerically higher.

Captures were not influenced in any regions by the separate or combined addition of *Z*13–18:OAc and *Z,Z*3,13–18:OH to the attractant blends.

Populations in the three regions studied here were similar with respect to being attracted to *E,Z*2,13–18:OAc by itself. However, the Tasmanian population differed significantly by failing to show a better response to the blend containing *E,Z*3,13–18:OAc. The remaining compounds studied influenced all three populations in a similar way, as far as trap captures were concerned. The different response of the Tasmanian population may point to the existence of a different pheromonal strain that has evolved locally, or it may mean that the population from which the present Tasmanian insects originated is different from the population in Central Europe. For the time being, a 100:3 *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc blend on rubber dispensers offers the best results for practical application in Central Europe and New Zealand, while *E,Z*2,13–18:OAc alone can be recommended in Tasmania.

Similar intraspecific variation in pheromone composition has already been reported in a number of cases for other Lepidoptera (Arn et al., 1983; Bailey et al., 1986; Guerin et al., 1984; Klun et al., 1973; Kochansky et al., 1975; Löfstedt et al., 1986).

In our experiments we never caught any other sesiid species. However, species other than *S. tipuliformis* have been reported elsewhere to be attracted to various mixtures of *E,Z*2,13–18:OAc and its isomers.

Three sesiid species were captured at different blends of *E,Z*2,13–18:OAc with *E,Z*3,13–18:OAc in Europe (Priesner et al., 1986). In North America, Snow et al. (1987) reported that when *Z,Z*3,13–18:OAc was added to *E,Z*2,13–18:OAc, the identified pheromone component of *Vitacea polistiformis* Harris (Schwarz et al., 1983), field captures were increased by three to seven times. In another field-screening conducted in central Georgia, captures of three sesiid species were improved by the addition of 2,13-octadecadienyl isomers to 3,13-octadecadienyl isomers (Snow et al., 1989).

The above cases demonstrate the joint action of 2,13- and 3,13-octadecadienyl isomers in the attraction of males of various sesiid species. Our findings represent a further example.

Daily Pattern of Trap Captures

We found that *S. tipuliformis* males were captured predominantly in the midafternoon. This corresponds with published observations on the occurrence of mating in this species (Balás and Sáringer, 1982). Observing the daily rhythm of calling behavior of *S. tipuliformis* in the laboratory under natural photoperiod, Buda and Karalius (1985) found two peak activity periods. Females called most intensively ca. 2 hr before sunset and, less intensively, around sunrise. The authors presumed that females call only in the afternoon in the field, and this is supported by the results of our study.

Behavioral Observations

Mixtures containing *E,Z*3,13–18:OAc added to *E,Z*2,13–18:OAc performed far better than *E,Z*2,13–18:OAc by itself, in terms of eliciting approaches and the time needed to complete an approach. This finding can explain why trap captures were dramatically increased by the addition of *E,Z*3,13–18:OAc in the field tests.

Yet more approaches were evoked by the blend containing 0.1% *Z,Z*3,13–18:OAc in addition to the binary mixture of *E,Z*2,13–18:OAc/*E,Z*3,13–18:OAc. The fact that trap captures were always higher in Hungary with similar ternary baits may reflect another aspect of the same phenomenon, even though these differences were not statistically significant. The addition of *Z,Z*3,13–18:OH or *Z*13–18:OAc, singly or together, to this ternary mixture did not have any further effect on approach behavior.

Since the highest level of landings was observed at the blend containing all five components, an additional, subtle behavioral effect of *Z*13–18:OAc and *Z,Z*3,13–18:OH can be surmised. It is interesting to note that the presence of both components seemingly is necessary. Since *E,Z*-, *Z,Z*3,13–18:OAc and *Z,Z*3,13–18:OH showed behavioral activity at very low percentages in the blends tested, they may not have been detected in previous work on the identification of the pheromone blend of *S. tipuliformis*. In this study we did not

have the opportunity to investigate whether these compounds are present in the pheromonal secretions of *S. tipuliformis* females.

Dead females attached to baits with a 100:3 mixture of *E,Z*2,13-18:OAc/*E,Z*3,13-18:OAc did not appear to affect the behavior of males. However, the involvement of nonchemical cues in close-range interactions between sexes just before copulation cannot be excluded.

In our study clear distinctions could be made between synthetic baits with respect to behavioral responses evoked from male *S. tipuliformis* under field conditions. Differences between baits in eliciting close-range approaches to the source corresponded well with differences found in trap captures. In contrast, differences between baits in evoking landing on the source were not reflected in numbers trapped. These differences might be important, however, when a different type of trap is used or when these blends are used for purposes other than trapping.

We conclude that the most complete sequence of sexual behavior is evoked from *S. tipuliformis* males by a 100:3:0.1:3:10 five-component blend of *E,Z*2,13-18:OAc/*E,Z*3,13-18:OAc/*Z,Z*3,13-18:OAc/*Z,Z*3,13-18:OH/*Z*13-18:OAc.

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LIMONENE INHIBITS ATTRACTION TO α-PINENE IN THE PINE WEEVILS *Hylobius abietis* AND *H. pinastri*

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Abstract—The field responses of *Hylobius abietis* (L.) and *H. pinastri* (Gyllenhal) (Coleoptera: Curculionidae) to various combinations of two host monoterpenes and ethanol were studied using baited pitfall traps. Both species were attracted to α-pinene, and when ethanol was added the attraction increased by 5–16 times. Limonene completely inhibited the attraction to α-pinene, even when the release rate of limonene was only about 1/50 that of α-pinene. The catches in traps with α-pinene and limonene as well as with limonene alone were similar in size to catches in empty control traps, i.e., no true repellent effect was demonstrated. When limonene was added to the combination of α-pinene and ethanol on old clear-cuttings, the catch of *H. pinastri* was completely inhibited while that of *H. abietis* was reduced by two thirds. On fresh clear-cuttings the inhibitory effect of limonene on the attraction to the α-pinene–ethanol combination was small or absent. Some aspects of host interactions are discussed as are practical implications regarding the choice of seedling material for planting and prospects of finding deterrents for protecting seedlings from pine weevil damage.

Key Words—*Hylobius abietis*, *Hylobius pinastri*, Coleoptera, Curculionidae, limonene, α-pinene, ethanol, olfactory orientation, attraction, inhibition, deterrent.

INTRODUCTION

The two Palaearctic pine weevil species *Hylobius abietis* (L.) and *H. pinastri* (Gyllenhal) are biologically very similar (Eidmann, 1974). Both species oviposit in roots of freshly killed or dying coniferous trees, and the larvae develop under the bark of the roots. Adult weevils feed mainly on the tender bark of

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stems and twigs of young conifers. Planted seedlings in reforestation areas are commonly killed by feeding pine weevils. Considerable economic losses are caused primarily by *H. abietis*, which is generally much more abundant than *H. pinastri*.

The olfactory orientation of *H. abietis* to its coniferous hosts has been the subject of several recent studies. Host monoterpenes and the biodegradation product ethanol have been found to be important attractants, while no pheromone has been demonstrated in *H. abietis* (Nordlander et al., 1986; Tilles et al., 1986a,b), except for a close-range mating stimulant present on the body surface of female weevils (Tilles et al., 1988). Nordlander et al. (1986) showed that weevils walking on the ground are able to locate underground roots suitable for oviposition by utilizing volatiles emanating from these roots and diffusing through the soil. A condensate of volatiles from stems of Scots pine, *Pinus sylvestris* L., was found to be highly attractive to *H. abietis* in a bioassay where the weevils responded by burrowing through a layer of sand towards the hidden odor source. When tested alone, the main component of this condensate, α-pinene, elicited about two thirds of the response obtained with the complete condensate, in which 14 monoterpenes were identified. Moreover, strong synergism between α-pinene and ethanol, when evaluated as attractants for *H. abietis*, has been demonstrated in field tests with baited pitfall traps (Tilles et al., 1986b; Nordlander, 1987).

The present study was initiated after discovering that the host monoterpene limonene can completely inhibit the strong attraction of walking pine weevils to α-pinene when both substances are released from baited pitfall traps (Nordlander, unpublished). Although limonene is known to repel or deter several insects associated with conifers (e.g., Rudnew and Smeljanez, 1969; Bordasch and Berryman, 1977), the observed effect on *H. abietis* and *H. pinastri* appears to be unusually conspicuous.

In previously published laboratory studies, *H. abietis* showed a neutral response or slight attraction to both (+)- and (−)-limonene (Selander et al., 1973, 1974; Nordlander et al., 1986). In the white-pine weevil, *Pissodes strobi* (Peck), Alfaro et al. (1980) found that limonene stimulated feeding at low concentrations but caused feeding inhibition when concentrations rose above a particular threshold. Limonene also has been found to be the most toxic of several host monoterpenes for the scolytid beetles *Dendroctonus brevicomis* LeConte, *D. frontalis* Zimmermann, and *Scolytus ventralis* LeConte (Smith, 1965; Coyne and Lott, 1976; Raffa et al., 1985). Moreover, there are strong indications that *D. brevicomis* preferentially attacks host trees with low concentrations of limonene (Smith, 1966; Sturgeon, 1979), and it has been shown that such trees are less resistant to attack than individuals with high limonene concentrations (Smith, 1969).

Limonene is present in variable amounts in Scots pine and Norway spruce, *Picea abies* (L.) Karst, which are the two main hosts of *H. abietis* and *H. pinastri* in northern Europe. In Swedish Scots pines limonene constitutes about 3–20% of the total cortical monoterpene content, and there is a distinct clinal pattern, with limonene proportions increasing with latitude (Yazdani et al., 1985; Yazdani and Nilsson, 1986). In Norway spruce investigated in Germany, the limonene content showed a bimodal pattern: high limonene levels were associated with low levels of β-phellandrene and vice versa (Heemann and Francke, 1977). Generally, the main cortical monoterpene hydrocarbon components of Scots pine as well as Norway spruce are α-pinene, β-pinene, 3-carene, myrcene, limonene, and β-phellandrene.

Monoterpene variation in conifers is largely genetically determined, and, in general, is not considered to be influenced much by environmental factors (e.g., Squillace, 1976). One exception, however, is the local, drastic increase in the proportions of limonene and certain other monoterpenes that sometimes occurs in response to infection by fungi associated with bark beetles attacking living trees (Raffa and Berryman, 1982, 1987). Mechanical wounding in the absence of the fungi does not appear to result in any major changes in monoterpene composition (Raffa and Berryman, 1982). However, mortal injuries, like girdling or felling, have been reported to increase the limonene–α-pinene ratio in *Pinus taeda* L. (Werner, 1972) while moisture stress has been found to decrease this ratio in *P. taeda* (Gilmore, 1977).

In this study the inhibitory effect of limonene on the attraction of pine weevils to host odors is investigated. The experiments deal with field attraction to various odor combinations employing the technique with baited pitfall traps described by Nordlander (1987). The effects on weevil attraction of adding limonene to either α-pinene or to the combination of α-pinene and ethanol were studied. In addition, different release rates of limonene were tested, and the effects of its two enantiomers were compared. It was also possible to compare the responses of *H. abietis* and its close relative *H. pinastri*. The olfactory behavior of the latter has not been studied previously.

Early field tests indicated that the relative catches on the various baits changed after the weevils had migrated in early June from 2-year-old clear-cuttings to fresh ones (i.e., cut during the previous winter). Therefore, comparative trapping was conducted (1) during May and June on 2-year-old clear-cuttings, where the newly emerged weevils were in a premigratory maturation feeding phase, (2) during June and July on fresh clear-cuttings, where arriving migrants were searching for oviposition sites, and (3) during August and September on 1-year-old clear-cuttings, where part of the adult population that had developed from eggs laid the previous summer had emerged and were feeding prior to hibernation.

METHODS AND MATERIALS

Odor-baited pitfall traps were used in field tests on clear-cuttings ca. 20–25 km N, NE, and E of Uppsala in central Sweden during 1984, 1985, and 1987 (Table 1). Each test compared the attractivity of two or four different treatments, consisting of odor baits placed in pitfall traps or of empty control traps. Traps were set out in blocks containing one representative of each treatment and with ca. 2 m between traps within a block and ca. 20 m between blocks. About 15 (13–17) such blocks were placed in a line across the clear-cutting. Differences between treatments were tested statistically with Friedman's test followed by a multiple-range test paralleling the Student-Newman-Keuls procedure (Zar, 1974) when four treatments were included, while a two-tailed Wilcoxon paired-sample test was used when only two treatments were compared.

The pitfall traps used in this study, with the exception of the extra control trap (C2) in the 1987 experiments, were all of the type illustrated in Nordlander (1987). The trap was constructed from a transparent polypropylene jar with a white polyethylene cap (height 121 mm, upper diam. 116 mm, lower diam. 95 mm). Eight 1-cm-diam. holes were equally spaced around the circumference of the jar, ca. 2 cm below its rim. The traps were placed with their holes just above ground level to allow walking weevils to enter. The C2 trap was made

TABLE 1. FIELD TESTS WITH ODOR-BAITED PITFALL TRAPS

Year	Test[a]	Age of clear-cutting	Trapping periods	No. of days in the field[b]	No. of replicates
1984	1a	2-year	May 17–23	6	15
	1b	fresh	June 7–20	13 (6 + 7)	15
	2a	2-year	May 23–30	7	17
	2b	fresh	June 15–28	13 (6 + 7)	15
	3	2-year	May 25–June 4	10 (5 + 5)	15
	4a	2-year	May 30–June 4	5	17
	4b	fresh	June 7–14 and June 20–27	14 (7 + 7)	15
	5	fresh	July 3–14	11 (6 + 5)	17
1985	6a,7a,8a	2-year	May 24–29	5	15, 13, 14
	6b,7b,8b	fresh	June 27–July 7	5	15
	6c,7c,8c	1-year	Aug. 26–Sept. 4	9 (4 + 5)	15
1987	9–12	2-year	May 6–July 1	56 (8 × 7)	15

[a] Treatments and results presented in Tables 3–5.
[b] Numbers in parentheses refer to intervals (days) at which traps were emptied and dispensers renewed.

from the same kind of jar but lacked the cap and the entrance holes of the other type of trap. This trap was placed with its rim level with the ground surface, like a conventional pitfall trap. All traps were filled with 0.15 liters of water.

The dispensers for volatile substances consisted of test tubes (depth 54 mm, inner diam. 8.5 mm) supplied with a strip of filter paper (50 × 8 mm, Munktell No. 3) reaching from the rounded bottom up to about 1 mm from the opening of the tube (the dispenser releasing limonene at a low rate lacked this filter paper). These dispensers were filled with either 1 ml (1984, 1985) or 1.5 ml (1987) of α-pinene, limonene, or a mixture of 95% α-pinene and 5% limonene, or with 2 ml (1984, 1985) or 3 ml (1987) of 70% ethanol. The volumes released of these substances were measured under conditions similar to those used in the field tests (Table 2). The baits consisted of an individual dispenser or a combination of dispensers containing different substances. The dispensers were suspended vertically with the openings ca. 2 cm below the center of the trap lid (illustrated and further described in Nordlander, 1987).

The following monoterpenes, supplied by Fluka AG, CH-9470 Buchs, were used in the field tests: (1*S*)-(—)-α-pinene (>97%, $[\alpha]_D^{20}$ −42 ± 3°), (*R*)-(+)-limonene (>98%, $[\alpha]_D^{20}$ + 115 ± 5°), (*S*)-(—)-limonene (≈97%, $[\alpha]_D^{20}$ − 90 ± 5°), and (±)-limonene (techn., = "dipentene").

RESULTS

The presence of limonene generally reduced the number of pine weevils attracted to α-pinene as well as to α-pinene and ethanol (Tables 3–5). As described below, this inhibitory effect varied with clear-cutting age, strength of attractive source, amount of limonene released, and pine weevil species. No sex-related differences in response were apparent for either of the pine weevil species. This conclusion was based on the use of contingency tables, when appropriate.

Sex ratios varied between clear-cuttings and between trapping periods on the same clear-cutting. However, no particular trends or patterns were observed regarding variation with clear-cutting age or time of year. In *H. abietis* the percentage of females varied between 40 and 65% in 23 test periods (tests 9–12 divided into two periods, tests 6c and 7c excluded); in 13 of these 23 cases the percentage ranged between 52 and 58%. The average percentage of females calculated for the total catch in all tests was 55% for *H. abietis* (N = 8509) and 52% for *H. pinastri* (N = 672).

Both pine weevil species were attracted to traps with an α-pinene dispenser. When (±)-limonene was added, trap catches were reduced to about the level of empty control traps. This inhibition of the attraction occurred when limonene was released from a separate dispenser located alongside the α-pinene

TABLE 2. AMOUNTS OF SUBSTANCES USED IN FIELD TESTS RELEASED FROM DISPENSERS PLACED IN PITFALL TRAPS DURING PERIOD WITH TYPICAL MAY TEMPERATURE CONDITIONS AND PERIOD WITH UNUSUALLY HIGH TEMPERATURES IN JUNE

Substance	Amount in dispenser (ml)[a]	No. of days in the field	Amount released per 5 or 7 days ($\bar{X}$ ± SD μl)[b]		Average amount released per day (μl)	
			Typical temp.[c]	High temp.[d]	Typical temp.[c]	High temp.[d]
70% Ethanol[e]	2	5	910 ± 33	1256 ± 178	182	251
(−)-α-Pinene	1	5	578 ± 116	646 ± 170	116	129
(±)-Limonene	1	5	264 ± 29	332 ± 53	53	66
(±)-Limonene	1[f]	5	18 ± 16	64 ± 36	4	13
70% Ethanol[e]	3	7	1844 ± 197	1968 ± 284	263	281
(−)-α-Pinene	1.5	7	806 ± 27	984 ± 149	115	141
(±)-Limonene	1.5	7	416 ± 35	538 ± 126	59	77

[a] Ethanol 2 ml and terpenes 1 ml correspond to baits used in tests 1–8, ethanol 3 ml and terpenes 1.5 ml correspond to baits used in tests 9–12 (see Table 1).
[b] $N = 5$; single dispenser in each trap.
[c] Typical temp.: mean air temperature for the five-day period (May 20–25, 1988) 9.4°C; for the seven-day period (May 20–27) 11.3°C.
[d] High temp.: mean air temperature for the five-day period (June 22–27, 1988) 19.8°C; for the seven-day period (June 22–29) 20.1°C.
[e] The exact amount of ethanol released is not known since 70% ethanol was used in accordance with previous studies (Tilles et al., 1986b; Nordlander, 1987).
[f] Dispenser lacking filter paper.

TABLE 3. PINE WEEVIL CATCHES ON BAITS WITH AND WITHOUT LIMONENE IN 1984 FIELD TESTS ON 2-YEAR-OLD AND FRESH CLEAR-CUTTINGS

Test	Baits[a]	Catch *H. abietis*[b]		Catch *H. pinastri*	
		2-year	fresh	2-year	fresh
1a, b	A	149 a	171 a	6	5
	L	0 b	10 b	0	0
	A & L	0 b	25 b	0	0
	C	2 b	23 b	0	2
2a, b	A	353 a	102 a	10	7
	L(low)	3 b	14 b	1	1
	A + L5%	3 b	18 b	4	0
	C	0 b	14 b	4	2
3	A	49 a		9	
	A & (±)L	7 b		1	
	A & (+)L	3 b		2	
	A & (−)L	2 b		0	
4a, b	A	83 a	85 a	5	1
	A + (±)L5%	6 b	21 b	3	0
	A + (+)L5%	25 ab	23 b	2	1
	A + (−)L5%	24 ab	22 b	1	2
5	A & E		434 a		24
	A + (±)L5% & E		335 b		16

[a] A = (−)-α-pinene; L = (±)-limonene (techn.); (±)L, (+)L, and (−)L = (±)-, (+)-, and (−)-limonene (97%); (low) = low release rate; +L5% = mixture containing 5% limonene; C = control (trap without bait); E = ethanol.

[b] Column figures followed by the same letter are not significantly different at the 5% level (tests 1–4: Friedman's test followed by a multiple comparison, test 5: Wilcoxon paired-sample test, two-tailed).

TABLE 4. PINE WEEVIL CATCHES WITH VARIOUS BAITS IN 1985 FIELD TESTS ON CLEAR-CUTTINGS EITHER 2 YEARS OLD, FRESH, OR 1 YEAR OLD

Test	Baits[a]	Catch *H. abietis*[b]			Catch *H. pinastri*
		2-year	fresh	1-year	fresh
6a, b, c	A	85	54	4	5
	A & L	0***	9*	1	0
7a, b, c	A	168	30	2	15
	A & E	785***	180***	6	42
8a, b, c	A & E	743	181	31	15
	A & E & L	240***	177NS	2**	13

[a] A = (−)-α-pinene; L = (±)-limonene (techn.); E = ethanol.

[b] Pairwise comparisons with two-tailed Wilcoxon paired-sample tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

TABLE 5. PINE WEEVIL CATCHES WITH VARIOUS BAITS IN 1987 FIELD TESTS ON 2-YEAR-OLD CLEAR-CUTTINGS

Test	Baits[a]	Catch *H. abietis*[b]			Catch *H. pinastri*[b]
		May 6–June 3	June 3–July 1	May 6–July 1	May 6–July 1
9	A	49	202	251 a	17 a
	A & L	3	3	6 b	1 b
	C	1	1	2 b	5 b
	C2	8	9	17 b	3 b
10	A	72	184	256 a	24 a
	A + L5%	8	30	38 b	2 b
	C	4	2	6 b	4 b
	C2	10	19	29 b	5 b
11	A	38	66	104 b	13 b
	A & E	458	729	1187 a	203 a
	C	2	0	2 c	3 b
	C2	3	3	6 c	3 b
12	A & E	512	861	1373 a	173 a
	A & E & L	185	318	503 b	6 b
	C	1	1	2 c	3 b
	C2	12	11	23 c	8 b

[a] A = (−)-α-pinene; L = (±)-limonene (techn.); +L5% = mixture containing 5% L; E = ethanol; C = control (trap without bait); C2 = control (trap without bait), open type.

[b] Column figures for the entire trapping period (May 6–July 1) followed by the same letter are not significantly different at the 5% level (Friedman's test followed by a multiple comparison).

dispenser as well as when a mixture containing 5% limonene and 95% α-pinene was released from a common dispenser (tests 1a, 1b, 2a, 2b, 9, 10). Traps baited exclusively with either a high- or a low-release limonene dispenser caught about as many weevils as the empty control traps and the traps with both α-pinene and limonene (tests 1a, 1b, 2a, 2b).

The relative catches of *H. abietis* in α-pinene-baited traps were usually considerably lower on fresh clear-cuttings than on 2-year-old ones seen in relation to the catches in both empty control traps and in traps baited with α-pinene and limonene (Table 6). During 1987, when pine weevil catches were recorded weekly from May 6 to July 1 on 2-year-old clear-cuttings (tests 9–12), no similar changes were observed in relative catches over time. (Owing to cold and rainy weather in May and June that year, only part of the pine weevil populations participated in the migration, which was observed to occur on June 6 and 22.) Thus, the observed decrease in relative catch for the α-pinene-baited traps on fresh clear-cuttings appears to have been attributable to the presence of fresh

TABLE 6. CATCHES OF *H. abietis* IN α-PINENE-BAITED TRAPS RELATED TO CATCHES IN TRAPS WITH COMBINATIONS OF α-PINENE AND LIMONENE AND IN CONTROL TRAPS

Test	Catch ratio[a]				Catch in A
	A/A & L	A/A + L5%	A/C	A/C2	
2-Year old clear-cuttings					
1a	>149		75		149
2a		118	>353		353
3	12[b]				49
4a		5[b]			83
6a	>85				85
9:(May 6–June 3)	16		49	6	49
9:(June 3–July 1)	67		202	22	202
10:(May 6–June 3)		9	18	7	72
10:(June 3–July 1)		6	92	10	184
Fresh clear-cuttings					
1b	7		7		171
2b		6	7		102
4b		4[b]			85
6b	6				54

[a] A = α-pinene; L = limonene; +L5% = mixture containing 5% L; C = control traps without bait; C2 = control traps without bait, open type.
[b] Ratio calculated using the mean catch of traps baited with (±)-, (+)-, and (−)-limonene.

host material and host odors competing with the baits rather than to a seasonal change in pine weevil behavior. Moreover, a comparison of the catches in the two types of control traps (C and C2; tests 9–12) did not reveal any tendency for interest in entering traps with holes to increase over time, which otherwise could have been an alternative for explaining the decreased A/C catch ratio.

In most of the tests an inexpensive (±)-limonene of technical purity was used. Limonene of higher purity (purum), consisting predominantly of either the (+)- or the (−)-enantiomer, was nevertheless used in three tests in order to check whether the inhibitory effect might be influenced by chirality or the presence of impurities. In test 4a, a racemic mixture of (+)- and (−)-limonene tended to have a stronger inhibitory effect than either of the enantiomers alone. However, in the two other tests (3, 4b), no difference in inhibitory effect between the racemate and the two enantiomers was found. The catch in traps with a combination of α-pinene and limonene tended to be larger in relation to the catch with α-pinene alone when the purer preparation was used. However, this relationship varied considerably between tests with the technical grade and was sometimes similar to that of the purer preparation (Table 6).

The combination of α-pinene and ethanol attracted many more pine weevils of both species than α-pinene alone. In tests 7a (on a 2-year-old clear-cutting) and 7b (on a fresh one), the catch of *H. abietis* with the combined bait was five and six times larger, respectively. The α-pinene–ethanol bait attracted even more weevils in relation to α-pinene alone in test 11, made on a 2-year-old clear-cutting; the combined bait caught about 11 times more of *H. abietis* and 16 times more of *H. pinastri*. No substantial differences in these relations were observed between the trapping periods before and after June 3.

When limonene was added to the α-pinene–ethanol bait the catch of *H. abietis* was reduced by two thirds in the tests on 2-year-old clear-cuttings (tests 8a and 12, both periods). The inhibitory effect of limonene on the catch of *H. pinastri* was much stronger (2×2 contingency table: $\chi_c^2 = 47$, $P < 0.001$); traps supplied with a limonene dispenser caught about as many *H. pinastri* as the empty control traps. On the fresh clear-cuttings, adding limonene had no effect on the catch of *H. abietis* in test 8b, while in test 5 the mixing of limonene with α-pinene (5:95) caused a small but significant decrease in trap catch. In test 5 the response of *H. pinastri* was similar to that of *H. abietis*, while no *H. pinastri* were caught in test 8b. Although few *H. abietis* were caught on 1-year-old clear-cuttings in late summer, it is clear from the data that limonene strongly inhibited attraction to α-pinene and ethanol in these presumably newly emerged weevils (test 8c).

DISCUSSION

That limonene inhibits attraction of pine weevils to α-pinene was discovered during the search for an effective standardized bait for trapping *H. abietis* (cf. Nordlander, 1987). Previous laboratory studies had indicated that limonene was one of several host monoterpenes eliciting digging behavior in *H. abietis* (Nordlander et al., 1986). Thus limonene was included as a potential attractant when first tested in the field (test 1a).

Since insects are commonly repelled or deterred by high concentrations of substances that are attractive or stimulatory in some way at lower dosages (e.g., Alfaro et al., 1980), the response to comparatively low release rates of limonene also was tested in the present study. However, when limonene was added to α-pinene, forming a mixture containing only 5% limonene, this was enough to completely or almost completely inhibit pine weevil attraction. The release rate from such a dispenser can be roughly estimated to have been around 2–3 μl limonene and 120 μl α-pinene per day at the prevailing temperature conditions during the tests, taking into account the proportions of the two substances and the lower volatility of limonene (cf. Table 2). This suggests that high concen-

trations are unlikely to have been responsible for the inhibitory effect of limonene observed in the field.

Substances inhibiting specific types of behavior, such as feeding or oviposition, are termed deterrents according to the designations of Dethier et al. (1960). This term might apply to limonene in the present case. However, it is not known whether limonene actually inhibits the normal response to α-pinene or if it is affecting some type of behavior counteracting the end result of the behavior induced by α-pinene. Any one of several behavioral changes, elicited either very close to the trap or at some larger distance from the odor source, could lead to the observed decrease in numbers of weevils entering the traps. Because these behavioral mechanisms are still unknown, I have preferred to use expressions such as "inhibited attraction" and "inhibitory effect" to account for what was actually observed in the field. These observations showed that the addition of limonene to the attractive odor source reduced the number of pine weevils captured; however, capture levels were no lower than those of empty control traps. Similarly, the low catch in traps baited with limonene alone was nevertheless about as high as the catch in control traps. Thus, a true repellent effect of limonene was not demonstrated.

Biological data on *H. pinastri* are scarce in the literature, and no information concerning responses to specific host substances in this species was available prior to the present study. The few published observations to date suggest that the life history of *H. pinastri* is roughly similar to that of *H. abietis* (Eidmann, 1974; Långström, 1982). However, a slight difference in habitat preferences has been found in some studies; in northern Europe it appears as if *H. pinastri* prefers moist sites dominated by Norway spruce, whereas *H. abietis* prefers drier Scots pine-dominated areas (Ozols, 1967; Långström, 1982). During the present study it was noted that *H. pinastri* was particularly abundant on wet parts of the clear-cuttings. This description of two biologically similar species with some modest differences in their habits is in agreement with the results of this study regarding responses to odors. Both pine weevil species were similarly attracted by α-pinene and by the synergistic combination of α-pinene and ethanol, while limonene more or less inhibited their attraction to these odor sources. However, this effect of limonene appeared to be more pronounced in *H. pinastri*. In test 12, for example, the addition of limonene to the combination of α-pinene and ethanol reduced the catch of *H. abietis* by only about two-thirds, whereas the attraction of *H. pinastri* was inhibited completely.

Host monoterpenes constitute essential cues for pine weevils in the process of locating the ephemeral breeding substrate they utilize (Nordlander et al., 1986). The monoterpenes play a role in defending the tree against various herbivores and pathogens. However, ovipositing females do not currently act as a selective force influencing the evolution of this defense system since living,

reproductive trees are not used as breeding material by these species. In contrast, weevil feeding may be a significant selective factor acting on the seedling. This should be the case if the genetically determined variation in monoterpene composition and other qualities affects (1) the risk of discovery by weevils, (2) palatability to weevils, or (3) tolerance of the seedling against damage. Little is known about whether levels of weevil-induced mortality differ between conspecific seedlings with different monoterpene compositions (see Selander and Kalo, 1979), but the findings regarding the effects of limonene in the present study indicate that such differences may exist. Further investigations in this area should be of practical interest also, inasmuch as the knowledge gained might help in determining the suitability of seedling material for planting. Particularly in clonal forestry, it should be worthwhile to consider any relationship between chemical composition of the seedling and damage caused by pine weevils.

In living natural host material, the relative amount of limonene is frequently higher than the 5% concentration found to be inhibitory in this study. For example, in cortical oleoresin samples taken from a large number of Swedish Scots pine populations (Yazdani et al., 1985; Yazdani and Nilsson, 1986), the amount of limonene was of the same magnitude as the amount of α-pinene, and in relation to the total monoterpene content the percentage of limonene was usually above 5% (3–20%). How then is it possible that pine weevil attraction to α-pinene can be completely inhibited at such low limonene levels whereas freshly cut or wounded Scots pines always appear to be attractive? One possibility is that limonene released as a part of the complete monoterpene bouquet from natural host material does not have the same inhibitory effect as it has together with only a single attractive monoterpene. It should not be assumed, however, that pine weevil attraction to natural host material cannot be inhibited by limonene. The degree of limonene-induced inhibition may vary—depending, for example, on whether the host material being considered consists of (1) either intact or injured seedlings used as food for adult weevils or (2) dying roots used as breeding material. In view of the urgent need to protect planted seedlings from pine weevil damage (e.g., Eidmann, 1981; Brunberg et al., 1986), the possibilities for using limonene, or related substances, for this purpose should be examined thoroughly.

Conventional insecticides and, to a lesser extent, some comparatively costly mechanical devices are used currently for seedling protection in northern Europe (e.g., Brunberg et al., 1986). No deterrents or repellents affecting pine weevils are in use. A turpentine distillate with deterrent or repellent effects on many insects, including *H. abietis*, has been tested recently by applying it to planted seedlings, but sufficient protection was not achieved (Eidmann, 1987). Thus, the finding reported here of a specific substance that can completely neutralize the strong attraction of another host monoterpene is encouraging. These results might be useful in future attempts to search methodically for substances capable

of reducing pine weevil damage, whether or not limonene eventually proves to be useful.

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ANTENNAL OLFACTORY RESPONSES OF BLACK TURPENTINE BEETLE, *Dendroctonus terebrans* (OLIVIER), TO BARK BEETLE PHEROMONES AND HOST TERPENES[1]

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Abstract—Electroantennograms (EAGs) were recorded from male and female black turpentine beetles, *Dendroctonus terebrans* (Olivier), exposed to bark beetle pheromones and host terpenes. The dose–response curves indicated similarities in the receptor mechanisms for both sexes for each compound. Antennal sensitivity was greatest to *endo*-brevicomin, which correlates with the importance of the compound in the behavior of the beetles. At above-threshold concentrations, EAGs were greatest to *endo*-brevicomin and frontalin, suggesting a large population of antennal receptors for these compounds. A large population of receptors would be expected for compounds that play such a significant role in this beetle's behavior. Beetles were also shown to have receptors that respond to the *Ips* pheromones, ipsenol, and ipsdienol.

Key Words—Electroantennogram, electrophysiology, black turpentine beetle, *Dendroctonus terebrans*, Coleoptera, Scolytidae, host odors, pheromones.

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INTRODUCTION

Payne et al. (1987) showed that male and female black turpentine beetles, *Dendroctonus terebrans* (Olivier), possess antennal olfactory receptors that respond to host terpenes and pheromones of the sympatric southern pine beetle, *D. frontalis*. They also showed that *D. terebrans* produce and respond behaviorally to some of the same pheromones as *D. frontalis* and proposed a relationship in which the pheromones produced by one species may function as kairomones for the other species. In order to further explore the ability of *D. terebrans* to detect pheromones of sympatric species, we conducted electrophysiological investigations using host odors, *D. frontalis* pheromones, and racemic mixtures of the sympatric *Ips* species pheromones ipsenol and ipsdienol (Silverstein et al., 1966; Renwick and Vité, 1972; Vité et al., 1976a, 1978).

METHODS AND MATERIALS

Beetles. Adult beetles of unknown age were collected from stumps or the bases of pine trees in the east Texas pine forest. Beetles were sexed (Godbee and Franklin, 1978), isolated in Petri dishes with moistened filter paper, and stored for 1–40 days at 6°C in a domestic refrigerator without photoperiodic control, prior to use in experiments.

Compounds. Nine beetle- and host tree-produced compounds were used in the study. The source and purity of each compound is listed in Table 1. Racemic mixtures of chiral compounds were used, even though chirality is important to

TABLE 1. SOURCE AND PURITY OF PHEROMONES AND HOST TERPENES USED IN STUDY

Compound	Source of supply	Purity (%)
endo-Brevicomin	Chem. Samp. Co.	99
Frontalin	BASF	99
Ipsenol	Borregard	81
Ipsdienol	Borregard	89
α-Pinene	Aldrich Chemical Co.	97
β-Pinene	Aldrich Chemical Co.	97
Verbenone	Chem. Samp. Co.	98
trans-Verbenol	Chem. Samp. Co.	99
Turpentine	Short path distillation of loblolly pine oleoresin	99

the response of sympatric bark beetles (Vité et al., 1976a,b, 1978). All compounds were diluted in nanograde pentane.

Electroantennograms. Electroantennogram (EAG) techniques (Dickens and Payne, 1977) were modified from earlier techniques (Schneider, 1957; Payne, 1970, 1975). Glass capillary Ag–AgCl electrodes filled with insect Ringer's solution (Barbosa, 1974) were used. Following prepuncture with a sharpened tungsten needle, the indifferent electrode was inserted into the head capsule; the recording electrode was inserted in the distal end of the antennal club. EAGs were displayed on a Tektronix 5223 digitizing oscilloscope and recorded on a Soltec x–y plotter.

Stimuli were delivered at 5-min intervals in increasing concentrations as 5 μl aliquots on filter paper (20 × 7 mm) placed into glass cartridges (75 mm long, 5 mm ID) and oriented toward the antennal preparation from ca. 1 cm. Stimulus duration was 2 sec in a 1 liter/min airflow filtered through activated charcoal. The initial depolarization upon stimulation was recorded as response to a given stimulus.

Serial dilutions of each compound were presented from the lowest to the highest concentration. The pentane solvent (5 μl on filter paper) was used as a control. The *D. frontalis* aggregation pheromone frontalin, at 5 μg, was used as the standard. Response to the standard was for males, $\bar{X} = 1.42$ mV $\pm$ SE $= 0.7$ mV, $N = 40$, and for females, $\bar{X} = 1.80$ mV $\pm$ SE $= 0.33$ mV, $N = 40$. Response to the control was for males, $\bar{X} = 0.31$ mV $\pm$ SE $= 0.03$ mV, $N = 40$, and for females, $\bar{X} = 0.37$ mV $\pm$ SE $= 0.05$ mV, $N = 40$. Response to the control was subtracted from the response to each stimulus. Response to the standard was recorded after the control and again after every two stimulations in a dilution series. Response to each dilution in the series was calculated as a percent of the mean of the two closest responses to the standard. This was done to normalize the data to control for variation between preparations and within preparations over time (Payne, 1975). Four beetles of each sex were tested for each compound. A single beetle may have been stimulated by more than one compound if the EAG elicited by the standard was greater than or equal to 1 mV.

All statistical analyses were computed using the Statistical Analysis System (SAS Institute Inc., Cary, North Carolina). The threshold of response to each compound was the minimum stimulus concentration at which, relative to the standard, the percent EAG to the stimulus was significantly greater ($P \geq 0.05$) than the percent EAG to the corresponding pentane control, as determined by the t test. Analysis of variance was used to compare percent response values between compounds for each sex at and above threshold. Means were separated using the least significant difference test. The t test was used to compare percent response values between sexes for each compound at each dose.

RESULTS AND DISCUSSION

All compounds used elicited EAG responses within the range of concentrations tested (Figure 1A–I). The similarity in shapes of the dose–response curves for male and female beetles to each compound suggest similar receptor mechanisms for both sexes. Significant differences in percent response values between the sexes did occur for turpentine at 5 μg, for α-pinene at 50 μg, and for *trans*-verbenol and verbenone at 0.5 μg on filter paper. These differences

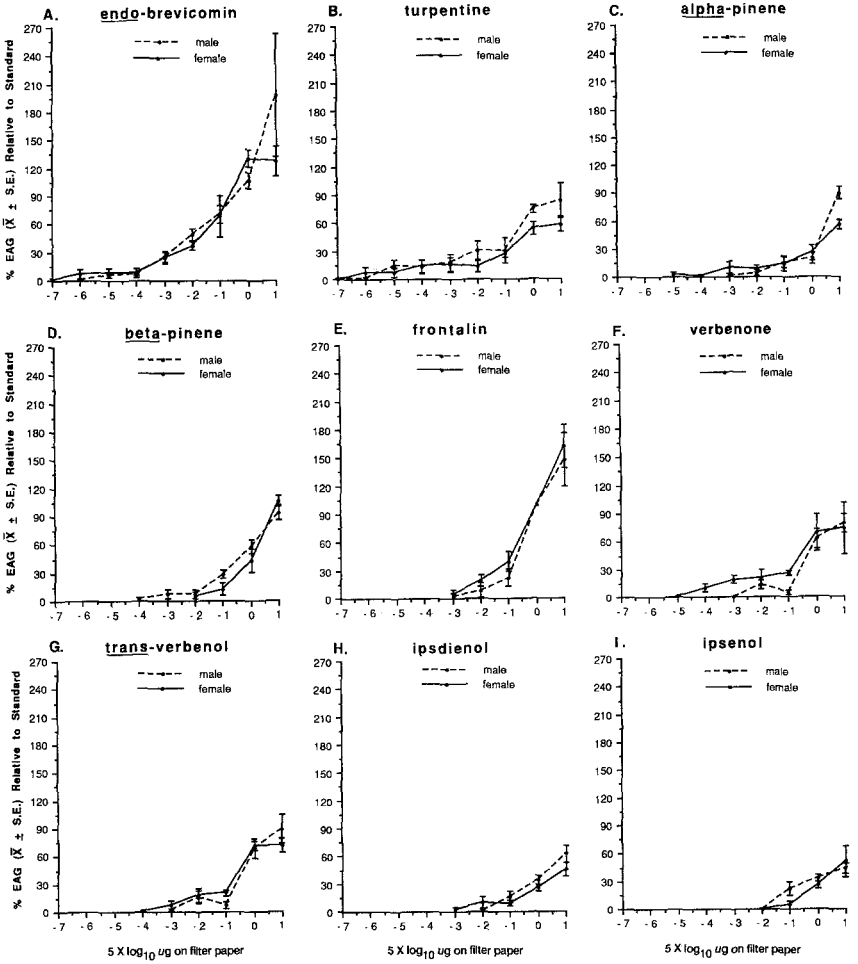

FIG. 1. Mean percent EAGs ± SE from *Dendroctonus terebrans* to pheromones and host terpenes, relative to the standard frontalin.

were not consistent over all concentrations and were likely the result of variability among the different preparations.

Response thresholds were lowest for *endo*-brevicomin (Table 2). The low threshold and wide concentration range to receptor saturation suggests that this compound may function in long-range orientation for *D. terebrans* (Dickens, 1981). Payne et al. (1987) found that *endo-brevicomin* was highly attractive to *D. terebrans* females in a pedestrian bioassay and when released with large quantities of turpentine in the field.

The higher thresholds for the other compounds suggest that they are more likely to play a role in intermediate- or short-range host orientation or synergism (Dickens, 1981).

At threshold doses and above (5 μg and 50 μg on filter paper), EAGs were greater for *endo*-brevicomin and frontalin than for the other compounds (Table 3), suggesting a larger population of receptors for these compounds than for the other compounds tested. This difference was significant ($P \leq 0.05$) at 5 μg, but not at 50 μg on filter paper. *endo*-Brevicomin is a male-produced *D. frontalis* pheromone that inhibits arrestment of conspecifics on mass-attacked trees (Vité and Renwick, 1971; Rudinsky et al., 1974; Payne et al., 1978; Richerson and Payne, 1979). Although *endo*-brevicomin is produced in only trace amounts by male *D. terebrans*, it is highly attractive to females of this species (Payne et al., 1987). The degree of antennal sensitivity and magnitude of response at higher concentrations, coupled with the strong female behavioral response, indicates that the *D. frontalis*-produced *endo*-brevicomin may function as a kairomone that benefits host-seeking *D. terebrans* females by directing them to weakened hosts.

Payne et al. (1987) reported that frontalin was highly attractive to male *D. terebrans* both in a pedestrian bioassay and in the field when released with large

TABLE 2. EAG THRESHOLDS OF *Dendroctonus terebrans* TO PHEROMONES AND HOST TERPENES

Compound	Female threshold	Male threshold
α-Pinene	10^{0}	10^{0}
β-Pinene	10^{0}	10^{-1}
endo-Brevicomin	10^{-3}	10^{-3}
Frontalin	10^{-1}	10^{-1}
Ipsdienol	10^{0}	10^{0}
Ipsenol	10^{0}	10^{0}
trans-Verbenol	10^{-2}	10^{0}
Turpentine	10^{-1}	10^{-2}
Verbenone	10^{-1}	10^{0}

TABLE 3. MEAN PERCENT EAG RESPONSE RELATIVE TO STANDARD[a] OF *D. terebrans* TO BARK BEETLE PHEROMONES AND HOST TERPENES

Concentration (μg)	Chemical	$\bar{X}$ ♀ Response ± SE	$\bar{X}$ ♂ Response ± SE
5	*endo*-Brevicomin	131.1 ± 9.0 a	107.2 ± 8.8 a
	Frontalin	100.0 ± 0.0 b	100.0 ± 0.0 a
	trans-Verbenol	71.5 ± 4.7 c	68.6 ± 10.3 b
	Verbenone	69.1 ± 19.7 cd	62.5 ± 10.3 b
	Turpentine	54.0 ± 6.7 cd	75.0 ± 4.4 b
	β-Pinene	43.0 ± 12.7 de	58.1 ± 7.0 b
	Ipsdienol	25.9 ± 3.9 e	33.9 ± 5.6 c
	Ipsenol	25.9 ± 3.4 e	33.5 ± 3.2 c
	α-Pinene	25.5 ± 7.9 e	20.0 ± 5.8 c
50	*endo*-Brevicomin	128.2 ± 16.3 ab	198.5 ± 64.0 a
	Frontalin	162.2 ± 22.4 a	147.7 ± 28.2 ab
	β-Pinene	106.3 ± 5.6 bc	94.3 ± 8.2 bc
	Verbenone	74.8 ± 7.8 cd	79.0 ± 9.9 bc
	trans-Verbenol	71.4 ± 7.8 cd	89.2 ± 15.7 bc
	Turpentine	58.3 ± 8.2 d	84.2 ± 18.4 bc
	α-Pinene	55.5 ± 5.2 d	89.7 ± 6.7 bc
	Ipsenol	51.2 ± 16.8 d	44.1 ± 5.4 c
	Ipsdienol	45.2 ± 7.2 d	62.7 ± 9.4 c

[a] 5 μg frontalin on filter paper.
[b] Means in the same column followed by the same letter are not significantly different at the 5% probability level according to the least significant difference test.

quantities of turpentine. Analogous results were reported by Phillips et al. (1989). Both studies found that this compound is produced and released by *D. terebrans* females. Female production, the magnitude of response at the higher concentrations, and the strong behavorial response by males implicate frontalin as a sex pheromone for *D. terebrans*.

The attraction of *D. terebrans* to turpentine is important in host finding. Turpentine-baited traps attract large numbers of *D. terebrans* (Fatzinger, 1985; Fatzinger et al., 1987). Thresholds for the monoterpenes α- and β-pinene were much higher than for turpentine. Turpentine consists of more than one volatile component (Renwick and Vité, 1970), and the lower threshold may result from the summation of receptor potentials from the simultaneous firing of receptors for the different components. Delorme (unpublished) found that *D. terebrans* males responded significantly to β-pinene in a pedestrian bioassay. The monoterpenes may play a role in close-range orientation for *D. terebrans*, as sug-

gested for *D. frontalis* (Payne, 1980). Receptor saturation for turpentine was reached at 5 μg on filter paper for both sexes, whereas response to α- and β-pinene was still increasing at 50 μg on filter paper. This suggests that receptors for α- and β-pinene also might be responsive to other monoterpenes in turpentine.

Verbenone, produced by male and female *D. frontalis*, inhibits arrestment of conspecifics on mass-attacked trees (Renwick and Vité, 1970; Payne et al., 1978). Phillips et al. (1989) reported that both sexes of *D. terebrans* produce verbenone during gallery construction, but did not discover a behavioral function for the compound. The high threshold and narrow range to saturation suggests that verbenone may function in close-range orientation or synergism (Dickens, 1981).

The dose–response curve for *trans*-verbenol has similar characteristics to that of verbenone, indicating that *trans*-verbenol also functions in close-range orientation or synergism. Payne et al. (1987) and Phillips et al. (1989) reported the production of *trans*-verbenol by both sexes of *D. terebrans*. Fatzinger et al. (1987) reported that *trans*-verbenol by itself was not attractive to *D. terebrans* in the field but had a synergistic effect when released with turpentine and ethanol. In contrast, Phillips et al. (1989) did not find any effect of *trans*-verbenol on the attraction of turpentine and frontalin to *D. terebrans*. Payne et al. (1987) reported that *D. terebrans* males were significantly attracted to *trans*-verbenol in a pedestrian bioassay.

Although ipsenol and ipsdienol elicited EAGs, the high thresholds and low responses at high concentrations (Figure 1H and I) indicate a relatively small population of receptors for these compounds. Billings (unpublished) found a significant decrease in field response of male *D. terebrans* to large quantities of turpentine when a mixture of 2% each of ipsenol, ipsdienol, and *cis*-verbenol was released with the host material. Delorme (unpublished) found that female *D. terebrans* response to ipsdienol was lower than to a pentane control in a pedestrian bioassay. It is known that olfactory interactions play an important role in resource partitioning among sympatric *Dendroctonus* and *Ips* species (Birch et al., 1980; Byers and Wood, 1980; Svihra et al., 1980; Flamm et al., 1987). The *Ips* pheromones may function to regulate host colonization by *D. terebrans* and sympatric *Ips*, especially *Ips calligraphus*, which can have overlapping ranges of distribution within the bole (Smith, 1957; Birch and Svihra, 1979; Flamm et al., 1988). The interaction of *D. terebrans* with *Ips* species warrants further investigation in the field.

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PHEROMONE RECEPTION IN TOBACCO BUDWORM MOTH, *Heliothis virescens*

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Abstract—Electrophysiological recordings from single olfactory receptor cells were carried out in the male tobacco budworm moth, *Heliothis virescens*. Recordings were made primarily from the sensilla trichodea type 1, which are located in the characteristic circumferential rows on the antennae. They possess the longest sensilla hairs as revealed by scanning electron microscopy (SEM). The sensory cells of these sensilla responded specifically to pheromones. Only three types of receptor neurons were found, each tuned to one of the female-produced components. The majority (58%) of the neurons were tuned to the major component (*Z*)-11-hexadecenal (Z11–16 : Al). Another large group (27%) responded specifically to stimulation with (*Z*)-9-tetradecenal (Z9–14 : Al). These two compounds are the most important components of the pheromones as judged by their influence on the behavioral responses of the males. The third type of neurons responded specifically to (*Z*)-11-hexadecen-1-ol (Z11–16 : OH), which may act either as a pheromone component or as an interspecific cue. None of the receptor neurons in the long sensilla trichodea responded specifically to the minor aldehyde components of the pheromone, which have subtle effects on behavior. Mixture experiments provided no evidence that minor components influence the receptor responses to the major components. Olfactory sensilla outside the crosswise rows were also characterized morphologically by SEM. Included in these were sensilla of different lengths, corresponding to a classification as s. basiconica and s. trichodea type 2. Electrophysiological recordings from these sensilla showed that they are involved primarily in host odor reception. However, a few of these neurons responded to pheromones.

Key Words—Tobacco budworm moth, *Heliothis virescens*, Lepidoptera, Noctuidae, olfactory receptor cells, single-cell recordings, EAG, pheromones, (*Z*)-11-hexadecenal, (*Z*)-9-tetradecenal, (*Z*)-7-hexadecenal, (*Z*)-9-hexadecenal, hexadecanal, (*Z*)-11-hexadecen-1-ol.

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INTRODUCTION

The tobacco budworm moth *Heliothis virescens* is a major pest insect affecting many species of crops, including cotton, tobacco, and corn. With the aim of developing a species-specific control technique, pheromones of *H. virescens* have been extensively analyzed by several research groups. Roelofs et al. (1974) and Tumlinson et al. (1975) independently identified the two major sex pheromone components, (Z)-11-hexadecenal (Z11–16 : Al) and (Z)-9-tetradecenal (Z9–14 : Al), from extracts of female abdominal glands and found that the natural ratio of about 16 : 1 (Z11–16 : Al to Z9–14 : Al) elicited responses of males. Subsequently, Klun et al. (1980a,b) identified five additional components: tetradecanal (14 : Al), (Z)-7-hexadecenal (Z7–16 : Al), (Z)-9-hexadecenal (Z9–16 : Al), hexadecanal (16 : Al), and (Z)-11-hexadecen-1-ol (Z11–16 : OH), in wash extracts of the glands. Although these components, when added to the two major components, did not seem to influence the male precopulatory behavior in laboratory bioassays, they significantly increased trap catches in the field.

Further detailed analyses of gland extracts and emitted volatiles have been carried out in combination with behavioral tests of the single constituents (Pope et al., 1982; Vetter and Baker, 1983; Teal et al., 1986). Teal and his colleagues found that all six aldehydes were emitted from both excised pheromone glands and from calling females, whereas the gland extract contained four additional alcohols. The ratio of the aldehydes emitted from the glands were 60% Z11–16 : Al, 18% Z9–14 : Al, 13% 14 : Al, 7.3% 16 : Al, 1% Z9–16 : Al, and 0.6% Z7–16 : Al. Behavioral studies in a flight tunnel demonstrated that all six aldehydes were important for the close-range reproductive behavior of the male, e.g., number of copulatory attempts, number of times the male reorients in close range, and the number of landing responses. These authors suggested that the alcohols were precursors for the unstable aldehydes and that they did not act as pheromone components (Teal et al., 1986). However, in another study it was suggested that addition of minor amounts of Z11–16 : OH to the three major aldehydes resulted in optimal trap catches of *H. virescens* (Ramaswamy et al., 1985). Another possible function suggested for minor components could be interspecific interruption. It might be that the Z11–16 : OH produced by female *H. subflexa* is responsible for the close-range disruption observed when *H. virescens* is released downwind from calling *H. subflexa* females (Teal et al., 1981a). Similarly, the Z9–14 : Al produced by female *H. virescens* could interrupt the pheromone attraction of another *Heliothis* species, *H. zea* (Klun et al., 1980b).

Generally, pheromone receptor neurons in insects, including Lepidoptera, bark beetles, and other species, seem to be of specialist types, i.e., they are tuned to one component of the pheromone blend (cf. Mustaparta, 1984). In Lepidoptera this is clearly shown in species that use one or two pheromone

components and is also the case for the major components in these species with more complex pheromone blends (Kaissling, 1979; Priesner, 1979). In the latter case, it has been difficult to find neurons tuned to the minor pheromone components, perhaps because they exist in a very low number (Priesner, 1979). However, it also has been suggested that minor components may modulate the responses of the receptor cells that are tuned to major components (O'Connell, 1985).

The present study sought to elucidate how the multicomponent pheromone blend, as well as interspecific chemical cues, are detected by single receptor neurons in *H. virescens*.

METHODS AND MATERIALS

Animals. Pupae of the tobacco budworm moth *H. virescens* from a laboratory culture were kindly provided by Dr. Max Angst, Ciba-Geigy, Basel, Switzerland. The sexes were kept apart as pupae and adults. The pupae eclosed at room temperature in closed containers, and the moths were fed sucrose in water ad libitum. Adult males were used for electrophysiological studies within eight days.

Scanning Electron Microscopy. Excised antennae were air-dried, mounted on aluminum stubs, and coated with a layer of platinum (200 Å). The preparations were examined at 15 kV, using a JSM 25 S microscope (Jeol).

The number of sensilla on the flagellum of both sexes was determined by counting the various types on every fifth segment, using scanning electron micrographs. With the exception of segments 10 and 40, the number was determined on one male and one female antenna. Segments 10 and 40 were counted in four males and three females to control the interanimal variation.

Electrophysiological Recordings. The moth was held in a Plexiglas holder. The head position was fixed by pieces of tape placed under the head and with Kerr utility wax (Kerr, Romulus, Michigan) and tungsten hooks (tungsten wire diameter 0.1 mm). The wax was gently pressed around the head to prevent movement while the base of the antenna was secured with hooks, one at the scape or pedicel and another at the third or fourth flagellar segment. In electroantennogram (EAG) recordings, the tip of the antenna (approximately segment 75) was fastened by hooks to wax, leaving most of the flagellum fully exposed for odor stimuli. According to standard methods, glass capillary electrodes (tip diameter 1 μm) filled with 3 M KCl were used for EAG recordings (Schneider, 1957; Kaissling, 1971). The recording electrode was inserted into one of the most distal segments (segments 75–81) and the indifferent electrode into a segment between the two hooks at the base of the antenna.

For single-cell recordings, the part from which the recordings were made

was firmly fixed by hooks onto the wax. Tungsten microelectrodes (Boeckh, 1962), with a tip diameter of less than $\frac{1}{3}$ μm were used (cf. Mustaparta et al., 1979). The recording electrode was inserted into the base of a sensillum until the extracellular impulse activity of the receptor neuron could be displayed (see Figure 4 below). The sensillum from which the recording was made was identified visually by observing either the positioning of the electrode into the hair directly or indirectly by the movement of the hair when the electrode was inserted (Leitz stereomicroscope, magnification 320×). At this magnification, hair length and position on the antenna are the only available visual cues.

The preamplifier used had an input resistance of 10 MΩ and noise level of 5 μVpp (low pass 3 KHz, high pass 10 Hz, 20 dB/DEK). The neural activity was monitored by a loudspeaker and displayed by a Hewlett Packard 181A oscilloscope with a 1809A four-channel vertical amplifier (100 mHz) and a 1825A time base/delay generator. Recordings were made by a Hewlett Packard 3964A instrumentation recorder and a Siemens-Elema Mingophon 3, by which analyses were made.

Stimuli. Pheromone components were kindly provided by Dr. Peter Beevor, Tropical Product Institute, London, England. The purity of the components was >99%, as reconfirmed by gas–liquid chromatography (Carlo Erba GLC with FID detector, using a 30-m × 0.25-mm fused silica DB 5 capillary column). Starting temperature was 60°C, increased by 8°C/min up to 220°C.

The following synthetic components were tested: Z11–16 : Al, Z9–14 : Al, Z9–16 : Al, Z7–16 : Al, 16 : Al, and Z11–16 : OH. In the screening tests, tetradecanal (14 : Al) was also included. About 50 mg of each component was diluted in 1 ml of hexane. The concentrations were adjusted to 50 mg/ml using GLC. Decade dilutions were made down to 0.5 ng/ml using Carlsberg pipets. Four series of the major and two series of the minor components were independently made in order to control the recording condition in each experiment. From each dilution, 200 μl was applied on a round (17 mm diam.) piece of filter paper (Schleicher and Schuell Roundfilter), the hexane was removed by a weak stream of pure nitrogen, and each filter paper was rolled, with clean forceps, and inserted into a glass cartridge (10 cm long, 4 mm diam.) that was closed with a plastic cap for storing. In addition to these test series, separate cartridges, containing 0.1, 10, and 100 μg respectively, were prepared for each compound. These were used for screening the sensitivity of each receptor cell to the various compounds. In order to minimize adaptation, the cell was initially screened to the lowest concentration (0.1 μg) of each compound, before the ordinary tests were made with the duplicate test series, alternately from low to high concentrations. At the end, screening tests were carried out using the high concentration cartridges.

Separate series were made for testing effects of pheromone mixtures. Two different mixtures were used: Z11–16 : Al plus the three minor aldehydes (Z9–

16 : Al, Z7–16 : Al, and 16 : Al), and Z9–14 : Al plus the same minor aldehydes. The mixtures contained the same amount of each compound. The effect of these test series was compared with that of the major components alone.

For control, syringes were prepared containing filter paper on which pure hexane was evaporated. The spontaneous activity varied from 0 to 10 spikes/0.5 sec. In most experiments, the activity during the 0.5 sec before stimulation (prestimulation activity) was recorded. Spontaneous activity is not subtracted from the response values.

Responses to host odors were determined with various plant materials (fresh leaves of grass and pot plants, pieces of fresh apple and orange) used as "green odors." Small pieces of the materials were placed inside the respective cartridges without obstructing the air flow.

Stimulation. The stimuli were applied via a syringe–olfactometer (Kafka, 1970; Mustaparta et al., 1980). The cartridge containing the odor was fastened to the outlet of a clean syringe which served to deliver the airflow through the cartridge. Seven milliliters of air was blown through the cartridge during a 0.5-sec stimulation period. The interstimulation intervals were 1 min for low stimulation intensities (below 1 μg amount in the cartridge) and 1–4 min at higher intensities. During the interstimulation period, clean air was blown over the preparation (0.3 cm/sec) and removed through openings in the experimental table connected to the ventilation system.

RESULTS

Scanning Electron Microscopy of Antennae. As expected from previous studies in the related species *H. zea* (Jefferson et al., 1970), the unbranched antennae of *H. virescens* exhibit characteristic sex differences (Figure 1). In contrast to the female antenna (Figure 1A), the male antenna contains sensilla (s. trichodea, type 1) with long, curved hairs, arranged in sequences of three to four circumferential rows. These extend from the scaled side toward the medial side (=leading edge, i.e., the side of the flagellum facing forward during flight) of the 50 proximal segments (Figure 1B and C). The length of these hairs (16–76 μm) increases from the midventral side toward the scales covering the dorsal side (Figure 1C). On the leading edge, other morphological types of olfactory sensilla are located. These also exist on the distal flagellar segments (No. 50–81) distributed on the nonscaled area. Besides the different spatial distribution (Figure 2), these olfactory sensilla also differ from type 1 by having shorter hairs (6–33 μm) and a different orientation angle (the type 1 sensilla are in a more upright position) (Figure 1B and C).

The olfactory sensilla outside the circumferential rows were classified as s. basiconica (10 μm) and s. trichodea type 2 (10–33 μm), which corresponds

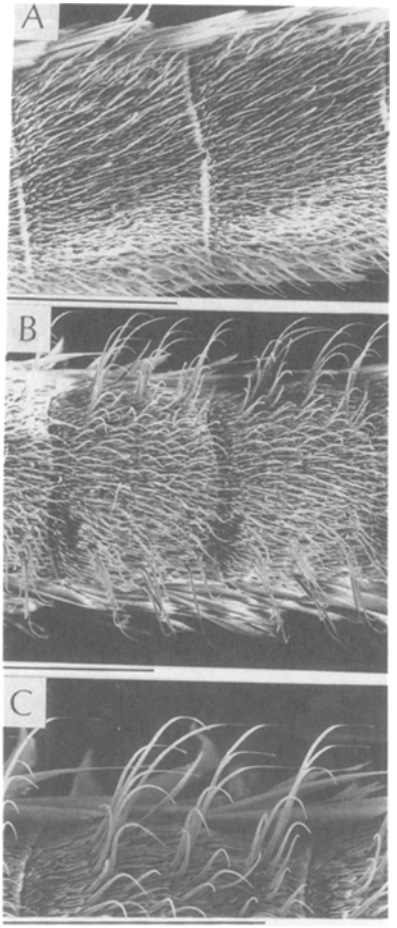

FIG. 1. Scanning electron micrographs demonstrating the sexually dimorphic antennae of *Heliothis virescens*. (A) Female antennae (450×); (B, C) male antennae (300×, 700×). The male-specific sensilla, s. trichodea type 1 (long, curved hairs) are located in three to four circumferential rows on each of the 50 proximal flagellum segments. Scale bar 100 μm.

to results from other moth species based on ultrastructure studies (Steinbrecht, 1973; Hallberg, 1981).

The female antenna lacks the long s. trichodea type 1 but does contain considerably more olfactory sensilla (17,000) than the male antenna (12,000) (Figure 2). The female olfactory sensilla are similar to those on the male antenna

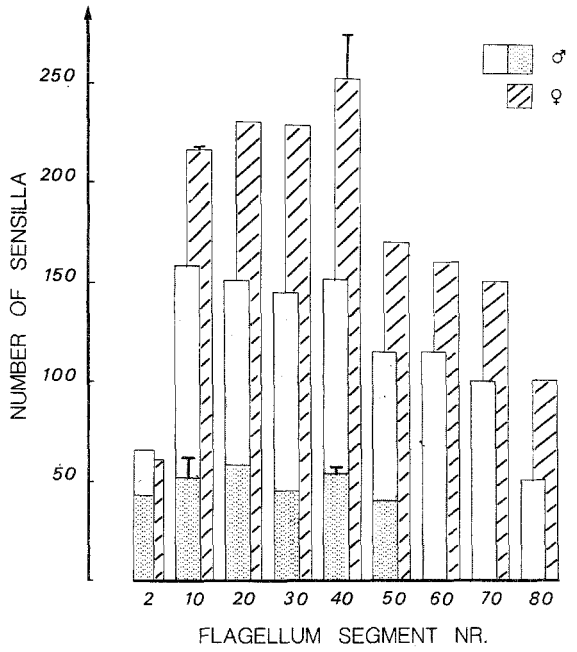

FIG. 2. Distribution of s. trichodea type 1 (dotted bars, present only in males), and s. trichodea type 2 plus s. basiconica (grey bars, males; hatched bars, females) in *H. virescens* on the approximately 81 flagellar segments. The histogram shows the number of sensilla on segments 2, 10, 20, etc. For segments 10 and 40 the standard deviation is indicated by vertical bars.

outside the crosswise rows (hair length 8–46 μm), and they are located on the nonscaled area of all flagellar segments.

Electrophysiology: Electroantennograms. The summated receptor responses (EAGs) to each of the five aldehydes tested and to the alcohol are shown in Figure 3. The male antennae were considerably more sensitive to the two major pheromone components, Z11–16 : Al and Z9–14 : Al, than to the other compounds. Moderate responses were obtained to three of the minor components (Z9–16 : Al, Z7–16 : Al, and Z11–16 : OH), whereas the response to the saturated 16 : Al was very weak at all concentrations tested.

Electrophysiology: Single-Cell Recordings. The data presented here from *H. virescens* males are based on recordings of 77 units responding to odors. Recordings were made for 36 additional units that could not be characterized as to sensory modalities.

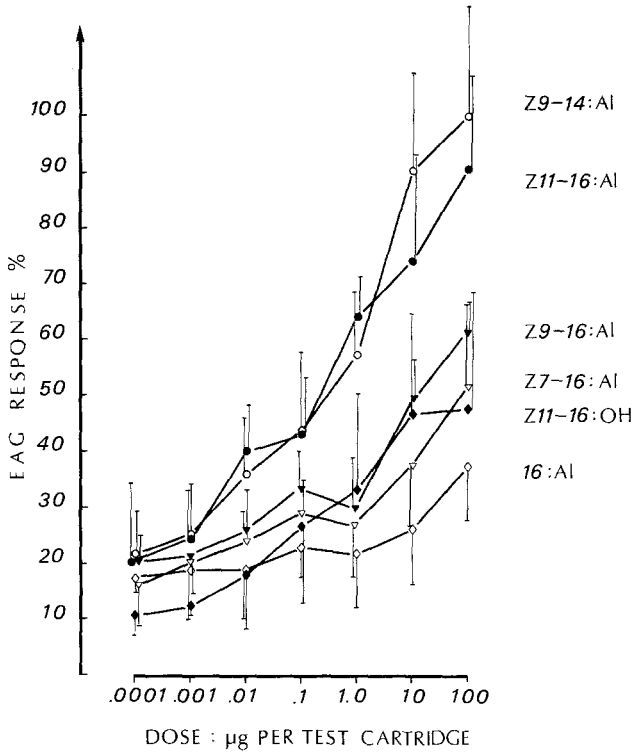

FIG. 3. Dose–response curves determined as EAG responses (mean values) to different concentrations of six pheromone components in *H. virescens*. The responses are calculated as percentage of a mean maximum response to Z9–14 : Al at 100 µg. Note that the most important components behaviorally, Z11–16 : Al and Z9–14 : Al, elicit much higher responses than the minor components. Standard deviation is indicated by vertical bars. Z11–16 : Al (N = 4), Z9–14 : Al (N = 4), Z9–16 : Al (N = 3), Z7–16 : Al (N = 4), 16 : Al (N = 4), Z11–16 : OH (N = 3).

The recordings often displayed impulse activity from two or three neurons. For further analysis, recordings showing distinctively the activity of one unit were selected. The intention was to record primarily from the male-specific s. trichodea type 1. However, in some instances included here, the recording position and sensilla movement could not be identified unequivocally. These latter recordings could have originated from s. trichodea type 1 or from other types of olfactory sensilla. Of 62 olfactory receptor cells studied in the crosswise rows, 51 responded to pheromones and 11 to green odors. The 51 recordings of pheromone responses included all cases where the electrode position was

shown to be at the base of the long s. trichodea type 1. There was some suggestion that the longest s. trichodea type 1 might differ functionally depending on the position in the crosswise rows, in that most Z11–16 : Al-responding cells were located towards the scale side with the longest sensilla hairs. The 11 green odor cells were located in an area more towards the medial side.

In recordings from the leading edge area, 15 olfactory receptor cells were identified, of which 12 responded to green odors and three to one pheromone component (with high threshold). None of the receptor neurons responded to both pheromone components and green odors. The 23 cells (11 in the crosswise rows and 12 in the leading edge area) responding to green odors showed different response spectra with regard to the various plant materials. In most cases the green odor cells responded both to fruits and green leaves (up to 100 imp/0.5 sec). However, some cells were activated solely by fruits or by green leaves. Furthermore, there were cells that responded only to one or two fruit odors, as well as cells that distinguished between odors of different fresh leaves.

The 51 pheromone-responding cells located in the rows could be divided into three groups, each specifically responsive to a particular pheromone component. There were 30 receptor neurons that responded specifically to Z11–16 : Al, 14 to Z9–14 : Al, and seven to Z11–16 : OH. The responses of one of the receptor neurons tuned to Z9–14 : Al is shown in Figure 4, demonstrating the phasic–tonic response pattern during the stimulation period of 0.5 sec (Figure 4A). Furthermore, stimulation with cartridges containing a high concentration of the key stimulus elicited responses that outlasted the stimulation period. This cell also responded to the minor component Z9–16 : Al, but only at the highest concentration (Figure 4D). In general, the pheromone receptor cells did not respond to components other than the key odor (Figure 4C and E, Figure 5), except for some Z9–14 : Al ($N = 4$) cells, which showed weak responses to Z9–16 : Al. Furthermore, the cells tuned to Z11–16 : Al and Z9–14 : Al had similar sensitivities, i.e., detectable responses to 0.001–0.01 μg and maximum responses (80–100 imp/0.5 sec) to 100-μg doses (Figure 6; Table 1). Slight desensitization, particularly appearing at low doses, was seen in a few experiments where preceding dose–response relationships had been completed on the same preparation.

The cells responding to the alcohol Z11–16 : OH exhibited much lower sensitivity than the aldehyde cells. Thus, the dose–response curves of these cells were shifted one to two log units to the right of those for the aldehyde cells. Furthermore, the responses to stimulation with 100 μg did not reach the maximum responses of the aldehyde cells. This is illustrated in Figure 6, showing the mean response values for the aldehyde cells and the alcohol cells.

None of the receptor neurons responded specifically or strongly to the minor components (Z9–16 : Al, Z7–16 : Al, and 16 : Al). In order to find out whether

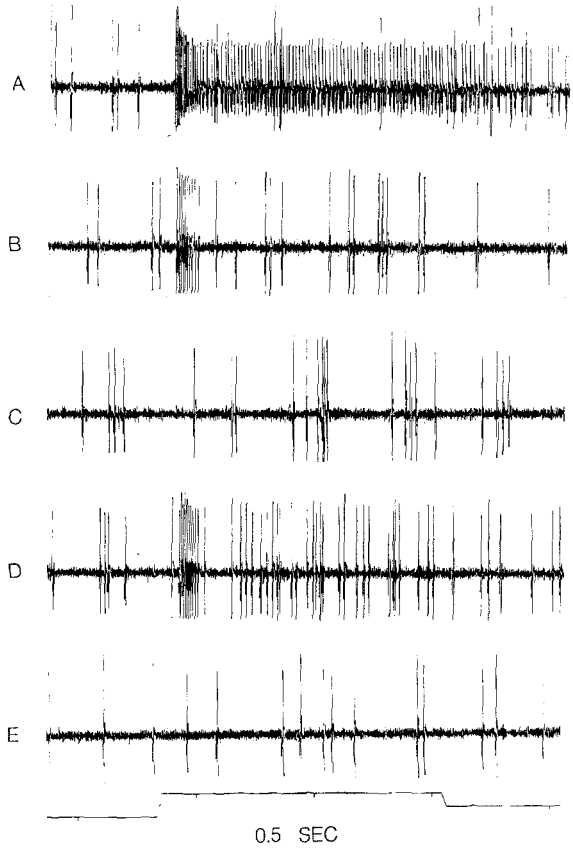

FIG. 4. *H. virescens* electrophysiological recordings of spike activity from a single olfactory receptor cell tuned to Z9–14 : Al. Responses to the key compound at a high (10 μg) and a low (0.1 μg) stimulation intensity are shown (A and B); as well as responses to high concentrations (10 μg) of the other major component Z11–16 : Al (C) and of two minor components (Z9–16 : Al and Z7–16 : Al in D and E, respectively). Note the analogous response to the Z9–16 : Al component.

the minor components may influence and/or modulate the responses of the cells tuned to the major aldehydes, these cells were also tested with mixtures. Figure 7 shows the result of one successful experiment where a Z11–16 : Al cell was tested at all concentrations, both for the key compound alone and for the mixture with equal amounts of the minor components added to the key component. Here, the dose–response curves for the key component and for the mixture overlapped completely. Similar results were obtained in experiments where the test series could not be fully completed. Both Z11–16 : Al and Z9–14 : Al cells were tested for their respective key compound alone and for that compound

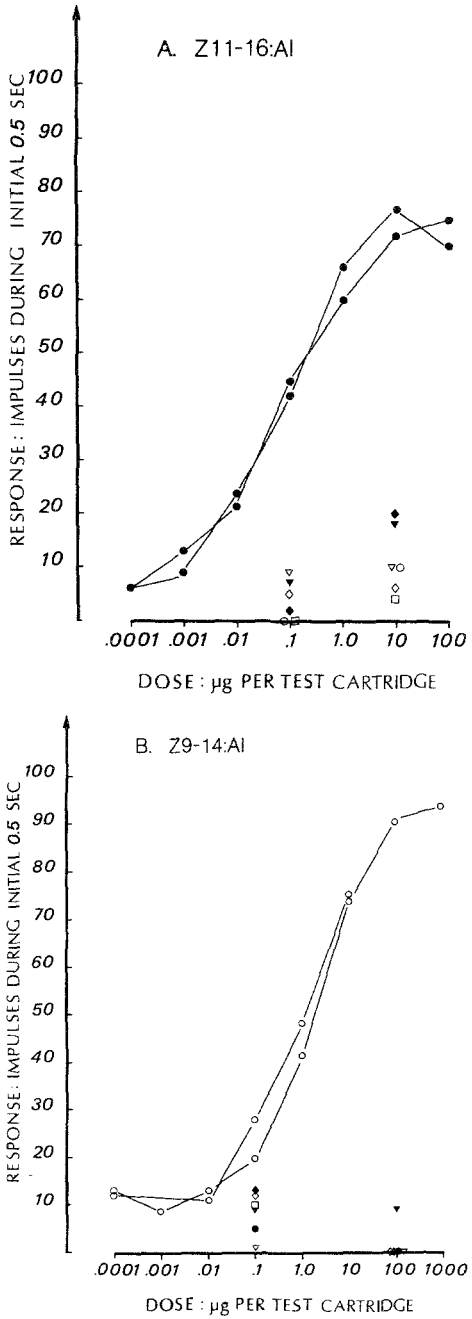

FIG. 5. Dose–response curves obtained from two receptor cells (A and B) in male *H. virescens*, each keyed to one of the major pheromone aldehyde components. The duplicated curves represent independently performed test series of the key compound. The prestimulation activity was low in the two experiments. Responses to the other test compounds are indicated by: ●, Z11–16:Al; ○, Z9–14:Al; □, 14:Al; ▼, Z9–16:Al; ▽, Z7–16:Al; ◇, 16:Al; ♦, Z11–16:OH.

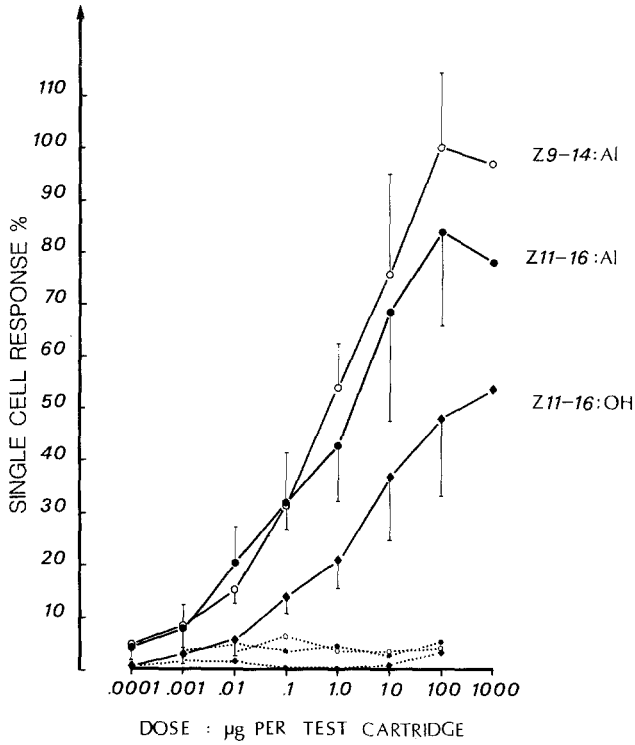

FIG. 6. Single-cell mean dose–response values in male *H. virescens* for the two major pheromone aldehydes Z11-16:Al ($N = 6$) and Z9-14:Al ($N = 4$) and the alcohol Z11-16:OH ($N = 4$). The responses are calculated as percentage of the mean response to Z9-14:Al at 100 µg. Standard deviations are indicated by vertical bars. Mean values of the prestimulation activity are shown by dotted lines and corresponding symbols.

together with the minor components, and mixture interactions were not observed.

DISCUSSION

The results show that in male *H. virescens* female pheromone components are perceived via specific olfactory receptor neurons in s. trichodea type 1 whereas host-plant odors generally seem to be perceived via other olfactory sensilla. It is interesting to note that the relative numbers of receptor cells tuned to the two major aldehydes, Z11-16:Al and Z9-14:Al (58% and 27%, respectively), correspond to their ratio in the pheromone blend (60% and 18%, respec-

TABLE 1. PHEROMONE RECEPTOR CELLS OF *H. virescens* CLASSIFIED ACCORDING TO KEY COMPONENTS Z11-16:Al, Z9-14:Al, AND Z11-16:OH[a]

	Dose (μg) per test cartridge							
Cell	0.0001	0.001	0.01	0.1	1.0	10	100	1000
Z11-16:Al								
1	•	•	••	••	•••	•••		
2	—	—	—	••	••	••••	••••	
3		—	•	•	••	•••	•••	
4		•	•	•	••	••	•••	
5	—	•	•	••	•••	••		
6		•	•	••	••	•••		
7				•	••	•••	•••	
8	—	—	—	•	••	••	••	
9		—	—	—	•	••	•••	
10		—		—		••	••	
11-24				•/••				
Z9-14:Al								
1		—	•	•	••	•••	••••	••••
2			—	••	•••	••••	••••	
3	—	—	•	••	••	•••	•••	
4				•	•	••	•••	•••
5		—	•	•	•	•••	••	
6		•	•	••	••	••		
7			—	•	—	•	•	•••
8		—	•	•		••••		
9					—	•	••	••
10		—		—	—	••	•••	
11				—	—	•	•	••
12-14				••				
Z11-16:OH								
1			—	•	•	••	••	••
2		—	—	•	•	••	•••	
3	—	—	•	•	••	••		
4		—	—	•	—	•	•	
5					•	••		
6					•••			

[a] The responses (imp/0.5 sec) are expressed as follows: — = indistinguishable from spontaneous activity, • = less than 30, •• = 30–60, ••• = 60–90, •••• = more than 90 imp/0.5 sec.

tively). It should be taken into consideration, however, that the present results apply solely to the s. trichodea type 1 in the crosswise rows, excluding possible pheromone-responding cells of s. trichodea type 2. Considering the relative filtering capacity of s. trichodea type 1, as well as the specificity of their recep-

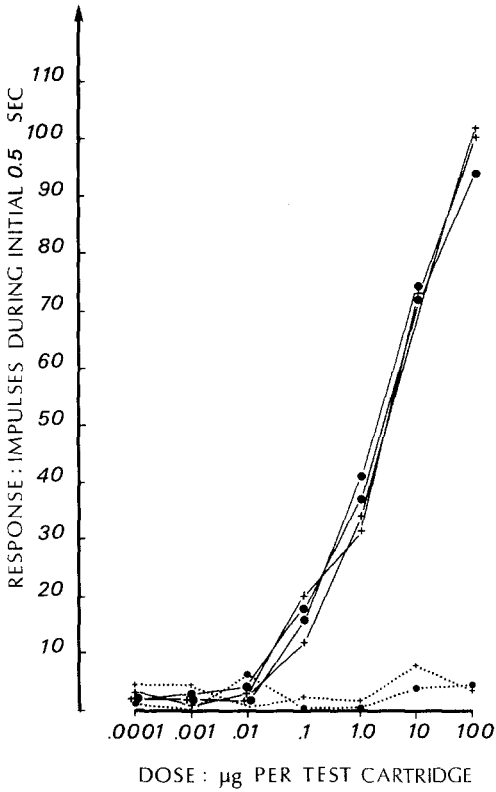

FIG. 7. Duplicated tests of the dose-related response of a single receptor cell in a male *H. virescens*, tuned to Z11-16:Al. •: The antenna exposed solely to the key substance. +: Exposed to the key substance mixed with equal amounts of the three minor pheromone components (Z9-16:Al, Z7-16:Al, and 16:Al). The prestimulation activity is shown by dotted line and corresponding symbols. Note that the addition of the minor compounds did not influence the responses to the key compound.

tor cells for the major components, it seems likely that the two major aldehydes play the most important role for long-distance attraction.

The three cells in the leading edge area, responding to Z9-14:Al, had a high threshold for this pheromone component and therefore may either be involved in pheromone perception or be specialized to other components not tested here (Almaas and Mustaparta, 1989). The sensilla located medially are obviously involved in host odor recognition since most units in this area responded strongly to green odors, either from leaves or fruits, and not to pheromones. Our intention is to subject these cells to further electrophysiological analysis.

The single-cell recordings imply that the various pheromone components are detected by separate cells, the majority of which are tuned to either one of the two major pheromone components Z11-16:Al or Z9-14:Al (Figure 5A and B). This is further evidenced by the EAG responses exhibiting highest amplitudes for these two aldehydes (Figure 3). That the Z9-14:Al cells to a minor extent reacted also to Z9-16:Al could be the manifestation of an "analogous" effect, where the Z9-16:Al molecules at higher concentrations are able to activate the membrane receptors for the Z9-14:Al molecule. This might adhere to the identical chemical sequence of the two molecules, located between the functional aldehyde group and the double bond in position 9, previously suggested to be the most important part of the pheromone molecule in noctuids (Priesner, 1979). This analogous effect may not be of biological relevance, since the cells are tuned to Z9-14:Al, which would act as the key stimulus as long as it is present in the blend. Influences of analogs on responses to the key pheromone have been tested in other species (Mustaparta et al., 1980). These tests suggest that no interaction takes place.

Among the pheromone receptor cells presented here, no cells were found specialized to the minor components. However, the EAG recordings showed moderate responses to these substances. Thus, it seems likely that some receptor cells for the minor components are present outside the crosswise rows.

The addition of minor components to any of the major pheromone aldehydes did not accentuate the response of these specialist cells. This implies that the subtle behavioral effect assigned to the minor compounds is not mediated by the specialist cells identified here. Thus, it speaks against the possibility that minor components modulate the responses of the major pheromone receptor cells, which is in accordance with the recent study by Akers and O'Connell (1988).

The demonstration of a third type of receptor cells, responsive to the alcohol Z11-16:OH, supports the possibility that this compound plays a role in male behavior. However, the results do not provide any clue as to whether this component plays any role in intraspecific communication (Ramaswamy et al., 1985) or interspecific communication. In other species, a relatively high number of receptor cells are tuned to interspecific inhibitors (Mustaparta et al., 1979) and show similar sensitivities and specificities as the pheromone-responsive cells (Mustaparta, 1986). Odorant-mediated isolation may be of general importance for *Heliothis* species. EAG responses in *H. zea* of similar amplitude were recorded for the interspecific inhibitor Z9-14:Al and for the major pheromone component Z11-16:Al (Christensen et al., 1989). The present results in *H. virescens* showed low sensitivity of the single cells to Z11-16:OH, leaving the possibility open that the responding cells are not tuned to Z11-16:OH as such, but rather are specialized to another *Heliothis* component not tested here, e.g., (Z)-11-hexadecen-1-ol acetate from *H. subflexa* (Teal et al., 1981b), acting as an interspecific cue.

The present results suggest that the pheromone components in *H. virescens* are perceived via specialist types of receptor neurons that convey pheromone information to the antennal lobe of the CNS via separate labeled lines of primary axons. A detectable threshold response of the major receptor cells, both as concerns EAG and single-cell recordings, appeared at doses of 0.001–0.01 μg. However, the absolute threshold responses are apparently lower, since the antennal lobe neurons respond strongly to 0.1 ng of Z11–16 : Al and Z9–14 : Al (T.A. Christensen, unpublished). This may be explained by the convergence of 3000–9000 receptor cells (Figure 2; up to three cells per s. trichodea) on 200–300 antennal lobe neurons. Similar observations concerning lower sensitivities of antennal lobe neurons than receptor cells have also been made in *Antherea* (Boeckh and Boeckh, 1979), *Bombyx* (Olberg, 1983), and *Manduca* (Christensen and Hildebrand, 1987).

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INHIBITION OF LARVAL SETTLEMENT BY NATURAL PRODUCTS FROM THE ASCIDIAN, *Eudistoma olivaceum* (VAN NAME)

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Abstract—Settlement bioassays with larvae of the cheilostome bryozoan *Bugula neritina* were used to isolate and purify antifouling compounds from the ascidian *Eudistoma olivaceum* (Van Name). Three inhibitors of larval settlement, two toxic and one nontoxic, were investigated. The toxic compounds accounted for approximately 0.4% of the organic fraction (on a dry weight basis) and were identified as eudistomins g and h, two of a number of alkaloids possessed by *E. olivaceum*. The nontoxic inhibitor of settlement was not characterized. Eudistomins g and h were effective inhibitors of larval settlement at concentrations as low as 0.5% of that present in the living animal.

Key Words—Natural products, eudistomins, settlement inhibition, ascidian, *Eudistoma olivaceum*, *Bugula neritina*, antifouling, chemical defense, alkaloid.

INTRODUCTION

Although the larvae of many marine invertebrates have the ability to settle epizoically on organisms, even on the soft or fleshy portions of these organisms (Ware, 1984; Davis, 1987; Davis and Wright, 1989), a number of sessile marine organisms are free of common fouling organisms. To reduce the likelihood of becoming fouled, organisms may employ several defense mechanisms includ-

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ing the production of mucus, sloughing of the epithelium, skeletal reinforcement, and the production of secondary metabolites (Bakus et al., 1986; Dyrynda, 1986; Davis et al., 1989). Indeed, natural products have been demonstrated to play significant roles in the inhibition of larval settlement (Standing et al., 1984; Rittschof et al., 1985, 1986), and, as increasing attention is focused upon their biological roles, their ecological significance will become clearer.

The colonial ascidian, *Eudistoma olivaceum* (Van Name) (Polycitoridae), is a common inhabitant of epifaunal communities in the shallow subtidal zone of southern Florida. *E. olivaceum* is most commonly found in enclosed waters, where settlement rates of fouling organisms are extremely high (C.M. Young, personal communication). Nevertheless, *E. olivaceum* is almost completely free of epizooites (Davis and Wright, 1989). We have previously provided evidence that natural products derived from *E. olivaceum* inhibit the settlement of the cheilostome bryozoan *Bugula nertina* (Davis and Wright, 1989). Here we report the chemical isolation and characterization of the settlement inhibitors.

METHODS AND MATERIALS

Preparation and Fractionation of Extracts. Colonies of *Eudistoma olivaceum* were collected from the shallow subtidal zone of the Indian River Lagoon, Florida, and frozen at −20°C until extraction. A methanol extract was prepared by maceration of the colonies (1270 g wet weight) in a Waring blender with 300 ml of methanol, followed by filtration through Whatman No. 1 filter paper. The ascidian residue was steeped in 100 ml of methanol for 1 hr and then returned to the blender and macerated again. The crude filtered extracts were combined and concentrated to dryness by distillation under reduced pressure to yield 48.8 g of a green extract (3.85% of the wet weight of the organism). The extract was then partitioned between water (200 ml) and 100-ml portions of ethyl acetate three times. The combined ethyl acetate fractions were concentrated by distillation under reduced pressure. During the first partitioning step, an oily green layer formed at the solvent interface. This layer was separated, concentrated to dryness and then partitioned between methanol–water (9:1 v/v) and heptane. The aqueous phase from this partitioning was combined with the previous ethyl acetate partitions to yield, after concentration, a combined fraction of 5.71 g (11.7% of the crude organic extract). This fraction was chromatographed under reverse-phase vacuum liquid chromatographic conditions on a C-18 stationary phase (Alltech C18 Bonded Davisil) using a step gradient of MeOH–water as eluent. The first fraction was eluted with 100 ml of water, the second with 100 ml of 50% MeOH, and the following two with 100 ml of 80% MeOH. Fractions 5–8 were eluted with 150 ml of 80% MeOH and fraction 9 with 100 ml of 100% MeOH. Fraction 10 was a combination of (1) 200 ml of

50% methanol and 50% methylene chloride and (2) 100 ml of 100% methylene chloride. All eluents were adjusted to contain 0.05% trifluoroacetic acid. The 10 fractions were tested to determine their ability to inhibit larval settlement. Fractions 6 and 7 showed the strongest settlement inhibition. Thin-layer chromatographic characterization of these fractions on analytical silica gel plates (EM Science) developed with chloroform–methanol (9 : 1) indicated that they both contained similar components including one with a retention factor (R_f) of 0.73 which turns bright yellow when sprayed with a solution of 2% vanillin in sulfuric acid. Four bands were observed when these fractions were further purified by preparative thin-layer chromatography [using chloroform–methanol, 9 : 1 as eluent on a Kieselgel 60 (2 mm thickness) preparative plate]. Settlement studies with the four bands indicated that band 1 was responsible for the settlement inhibition. Further purification of this fraction by HPLC was carried out on a VYDAC C-18 protein and peptide column with acetonitrile as eluent. The major component (N75%) was found to be spectroscopically identical to eudistomin h while the minor component (N20%) was found to be spectroscopically identical to eudistomin g. These compounds accounted for approximately 0.04% of the wet weight of *E. olivaceum* and 0.4% of the dry weight. Both of these compounds have been characterized previously (Kobayashi et al., 1984).

Settlement Bioassay. Larvae of the arborescent cheilostome bryozoan *Bugula neritina* were obtained by exposing reproductive colonies, collected from the Indian River Lagoon, to bright light. Larvae were used in settlement bioassays within 1 hr of their release. Batches of larvae (20–40) were pipetted into 5-ml glass stender dishes containing 3 ml of the same seawater in which the adult colonies had been kept. After the addition of larvae, the quantity of seawater was made up to 3.5 ml in all dishes.

Squares of polystyrene (1.5 × 1.5 cm) were cut from Petri dishes (Labtek) and carefully coated with fractions of *E. olivaceum* solubilized in methanol. The concentration of fractions used in two experiments was set to approximately 5% of the natural concentration in the living ascidian, while in a further experiment concentrations were set at between 12.5% and 0.05%. Concentrations were calculated on a surface area basis (Davis and Wright, 1989). At all dilutions, 150 μl of methanol was added to the treatment and control squares of polystyrene; controls received solvent only. The solvent was allowed to evaporate off the polystyrene in a fume hood prior to beginning trials. Treated and control polystyrene squares were added to dishes, paired with respect to the number of larvae they contained. Dishes were transferred to a 24°C constant temperature cabinet and kept in absolute darkness. After 24 hr the number of larvae in each of four categories was determined: settled on the polystyrene, settled on the glass dish, inactive, or swimming. Only data in the settled on polystyrene and inactive categories were considered. The majority of larvae fell into these two categories.

Statistical Analyses. Comparisons between experimental and control dishes were made with a paired sample t test when data were normally distributed, otherwise a Wilcoxon paired rank test was used (Sokal and Rohlf, 1969). The percentage of larvae settled and inactive were arcsine transformed prior to doing the parametric test.

RESULTS

In laboratory trials, 45–75% of *B. neritina* larvae in each dish settled onto control (methanol-coated) pieces of polystyrene within 24 hr. In contrast, many of the initial 10 fractions of ascidian extract significantly inhibited the settlement of larvae (Figure 1A). Notably, fractions 6 and 7 were the only two that completely inhibited larval settlement and rendered all larvae inactive (Figure 1B). Indeed, most of these larvae were dead. Fraction 3 was unusual in that it significantly inhibited the settlement of larvae but did not render these larvae inactive; most continued to swim in the presence of this fraction.

Among the controls, larvae that were inactive rarely exceeded 20%, but in the presence of fractions 4–8 over 90% of the larvae in dishes were inactive. Settlement was correspondingly lowest in these fractions. Examination of all fractions with thin-layer chromatography revealed that the bright yellow compound ($R_f = 0.73$) found in fractions 6 and 7 was also present in trace amounts in fractions 4, 5, and 8. This compound, later identified as a potent inhibitor of larval settlement, was a combination of eudistomin g and h.

Compounds in fraction 7 were separated by preparatory thin-layer chromatography, yielding four bands ($R_f = 0.73$, 0.52, 0.11, and 0.04). Only the two bands with the highest R_f values (bands 1 and 2) were tested in settlement assays as bands 3 and 4 were considered the products of degeneration. Bands 1 and 2 significantly inhibited larval settlement, with band 1 exhibiting the highest activity (Figure 2). Further purification of band 1 with HPLC yielded eudistomin g and h in a 1 : 3 ratio.

In settlement trials, eudistomin h significantly inhibited larval settlement at a concentration of 5% of that found in the living ascidian. That is, from an estimated concentration of 44.5 $\mu g/cm^2$ in the living ascidian, 2.2 μg was applied to each square centimeter of polystyrene in the settlement trials. Eudistomin g, although present in a lower concentration in the living ascidian, was also tested at this concentration to determine the relative potency of these two alkaloids. At equal concentrations, both eudistomins appeared equally effective at deterring bryozoan larvae from settling. In the presence of eudistomin g, only an average of 3.2% (SE = 1.45) of bryozoan larvae settled onto the polystyrene, while 72% (SE = 5.4) settlement was observed on the solvent controls ($W = 55$, $P < 0.01$, $N = 10$). Similarly, only 2.6% (SE = 0.9) of the bryozoan

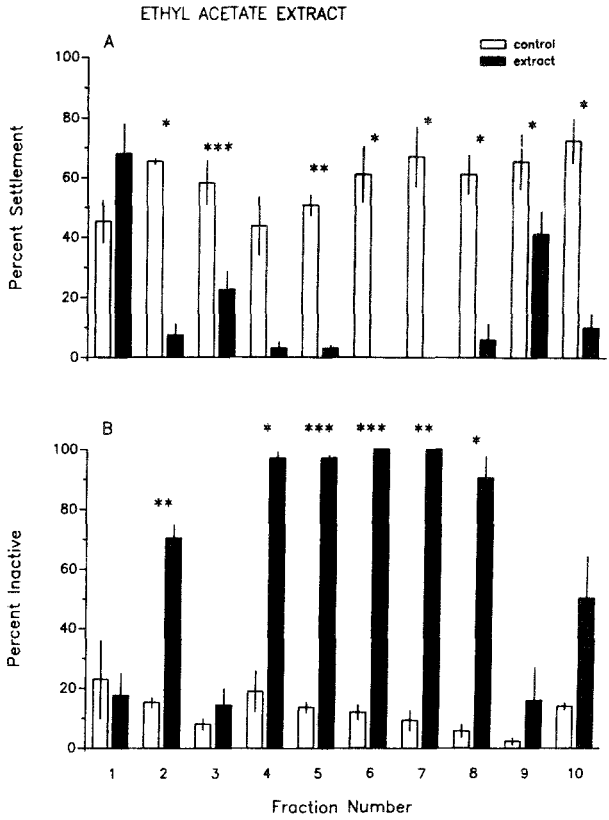

FIG. 1. Percent (A) settled (on polystyrene) and (B) inactive larvae of the bryozoan, *Bugula neritina* in 24-hr settlement assays. The highest fraction numbers represent the most polar fractions. Concentrations of all fractions are at 5% of that in the living ascidian. Bars are means of three to eight replicates from paired control (open bars) and treatment (closed bars) dishes (fraction 3, $N = 8$; fraction 8, $N = 6$; all other fractions, $N = 3$). Error bars are standard errors. Levels of significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

larvae settled on eudistomin h applied to polystyrene, while 82% (SE = 5.2) settled in the controls ($W = 28$, $P < 0.05$, $N = 7$).

Fraction 3, the nontoxic fraction (see above), was chromatographed on a C-18 stationary phase (Alltech C18 Bonded Davisil) using a step gradient of MeOH–water as eluent. Ten bands were collected but only band 8 caused significant inhibition of larval settlement (Figure 3). As expected from the earlier settlement trials, percentages of inactive larvae were low and not significantly different in experimental and control trials ($t = 2.29$ $P > 0.05$). Examination

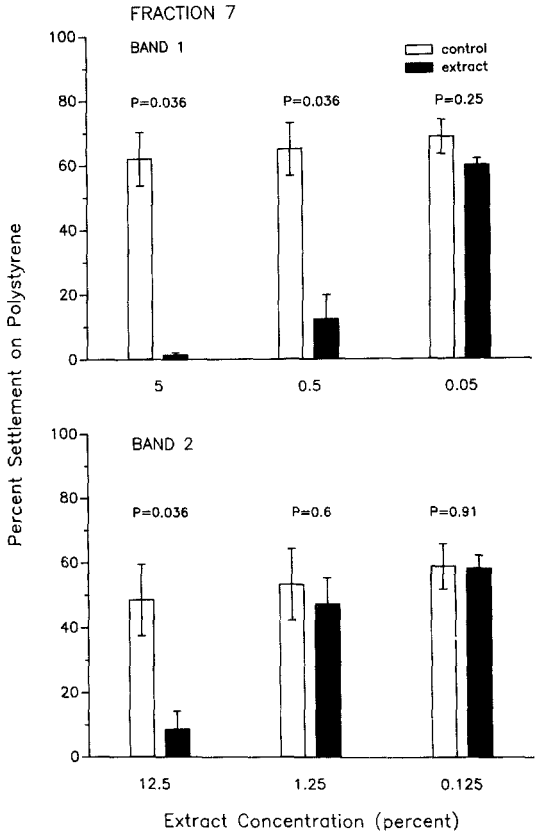

FIG. 2. Percent settlement of *Bugula neritina* with two bands (obtained with PTLC) of fraction 7. Bars are means of six replicates and error bars are standard errors. Concentrations of bands varied between 12.5% and 0.05% of that in the living ascidian. Levels of significance same as Figure 1.

of band 8 with TLC and NMR revealed a large number of compounds, but the compound(s) responsible for the inhibition of settlement were not characterized further.

DISCUSSION

We have provided evidence that *Eudistoma olivaceum* possesses toxic and nontoxic inhibitors of bryozoan settlement. The toxic metabolites, eudistomins g and h, were present in relatively high concentrations and their effect on bryozoan larvae lends support to our suggestion that eudistomins were responsible

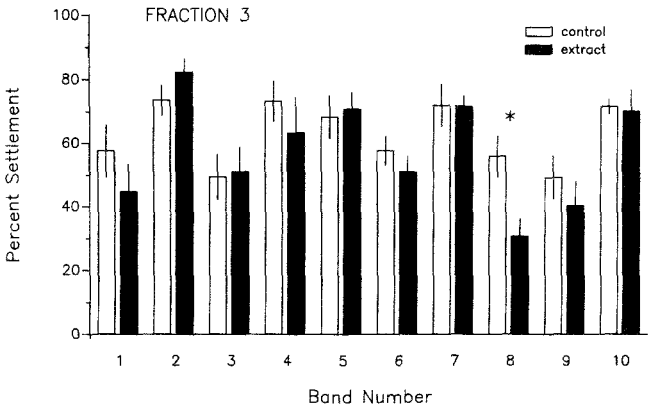

FIG. 3. Percent settlement (on polystyrene) of *Bugula neritina* with 10 bands from fraction 3. Concentration of all bands are at 5% of that in the living ascidian. Bars are means of three to eight replicates from paired control (open bars) and treatment (closed bars) dishes (bands 6 and 8, $N = 8$; band 9, $N = 6$; all other bands, $N = 3$). Error bars are standard errors. Levels of significance same as in Figure 1.

for the near epibiont-free test of this ascidian (Davis and Wright, 1989). Other potential mechanisms of inhibition, such as an acidic tunic or high concentrations of vanadium, as proposed by Stoecker (1978), were ruled out as important inhibitors of larval settlement in *E. olivaceum* (Davis and Wright, 1989).

Surface wettability (i.e., the tendency to induce spreading of a liquid on a surface) modifies the settlement of the bryozoan *Bugula neritina*; unfilmed polystyrene has high surface wettability and is highly preferred by these larvae (Mihm et al., 1981; Rittschof, personal communication). Changes in wettability may have occurred on the addition of eudistomin g and h to polystyrene, but the death of larvae on exposure to these compounds indicates that these alkaloids are responsible for the inhibition of larval settlement. However, it is unclear whether the nontoxic inhibitor of larval settlement is acting directly on the bryozoan larvae or merely altering the surface wettability of the polystyrene in the settlement trials.

Low concentrations of eudistomin g and h are apparently water soluble (Davis and Wright, 1989) and deter the settlement of bryozoan larvae, but it is difficult to compare directly the potency of these alkaloids with other antifouling compounds. The disparate susceptability of invertebrate larvae to compounds from different invertebrates (Dyrynda, 1985) and the differences in bioassay methodology (e.g., Standing et al., 1984; Rittschof et al., 1985) suggest that any such interpretations be made cautiously.

It remains unclear whether *E. olivaceum* synthesizes antifouling com-

pounds de novo or if microorganisms, either associated or ingested, produce it. The role of microorganisms in the antifouling activity associated with *E. olivaceum* is being examined currently (Davis and Essich, work in progress).

Twenty eudistomins have been isolated from *E. olivaceum* and several of these show potent antiviral activity (Kobayashi et al., 1984; Rinehart et al., 1984, 1987; Kinzer and Cardellina, 1987). Eudistomins g and h showed only "modest activity" against *Herpes simplex* virus, type 1 (Kobayashi et al., 1984), yet showed the most potent antifouling activity in our assays. Assuming that other eudistomins were present in the samples collected from Indian River Lagoon, their ecological role (if any) is not clear.

In our settlement bioassays we used only a single species, the larvae of the cheilostome bryozoan *Bugula neritina*. Species-specific effects on larvae were documented by Dyrynda (1985) who found that larvae of one bryozoan would not settle and were often killed in the presence of small pieces removed from sessile invertebrates, particularly sponges, while larvae of a congeneric bryozoan were unaffected. More recently, natural products from coelenterates have shown specificity in antifouling activity toward invertebrate taxa. Compounds that inhibited the settlement of bryozoans had no impact on barnacle larvae, while barnacle inhibitors did not influence bryozoans (Rittschof et al., 1988). It may be premature to ascribe an antifouling role to eudistomin h until we have data on its effect on the larvae of other invertebrate taxa. Examining the influence of eudistomin h on a variety of invertebrate taxa is the next step in our research.

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LARVAL RELEASE IN BRACHYURAN CRUSTACEANS
Functional Similarity of Peptide Pheromone Receptor and Catalytic Site of Trypsin

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Abstract—Studies of crab egg hatching and larval release behavior in the crab, *Rhithropanopeus harrisii*, generated a model describing the process. In the model, carboxyl terminal arginine peptides serve as pheromones that synchronize larval release. In response to the peptides, the female performs stereotypic larval release behavior and casts larvae into the water column. The peptides originate from trypsin-like enzymatic activity as part of the egg-hatching process. Hatching can be simulated experimentally by incubating ovigerous crabs in either bovine or porcine trypsin. The female performs the larval release behavior. Eggs detach from the female, and immobile larvae hatch prematurely. Preincubation of trypsin with trypsin inhibitors eliminates these effects. Approximately nanomolar concentrations of five different polypeptide trypsin inhibitors evoke the female's larval release behavior. Because both peptides and trypsin inhibitors evoke larval release behavior and because trypsin inhibitors bind to both the peptide receptor and the enzyme with high affinity, the receptor binding site and trypsin catalytic site must be very similar. A relationship between the binding site of a peptide receptor and the catalytic site of trypsin is postulated. The difference may be substitution by a basic amino acid for the catalytic site serine. Molecular graphics modeling indicates that all necessary conditions for receptor binding can be met by substitution with lysine for the active site serine in the trypsin catalytic site. This substitution eliminates catalytic activity, maintains the binding affinity for trypsin inhibitors, and increases binding strength for peptides.

Key Words—*Rhithropanopeus harrisii*, crab, Crustacea, larval release pheromone, peptide pheromone, trypsin.

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INTRODUCTION

Brachyuran crustaceans have highly rhythmic, synchronous patterns of larval release, often timed by environmental variables such as light and tides (Forward, 1987). Based upon past research with the crab *Rhithropanopeus harrisii* (Forward et al., 1982, 1987; Forward and Lohmann, 1983; Rittschof et al., 1985, 1989), we propose the following general model for larval release. Ovigeous females incubate attached eggs for a number of days or weeks, depending on the species. The actual timing of egg hatching is controlled by the developing embryo. Shortly before hatching, the eggs swell and the outer egg membrane or chorion breaks, leaving a fragile inner egg membrane around the embryo. At the specific time of hatching, pheromones are either secreted from storage locations within the embryos or are generated by proteolytic digestion of the egg membranes by enzymes released from the embryos. The pheromones cause the female to perform vigorous abdominal movements (pumping response) that mechanically disrupt the eggs, synchronize hatching, and propel the larvae into the water column.

In *Rhithropanopeus harrisii* the pheromones are two- to five-amino acid oligopeptides (Rittschof et al., 1985). Synthetic di- and tripeptides containing one or two neutral amino acids and a carboxyl terminal arginine mimic the pheromones because they evoke the female's larval release behavior (Forward et al., 1987; Rittschof et al., 1989).

The proposed model specifies that peptide pheromone generation occurs either by proteolytic cleavage of the egg membrane proteins or through internal proteolytic activity followed by the release of pheromones by the embryos at the time of hatching. An initial objective of the present study was to differentiate between these alternatives. Induction of the pumping response and premature release of larvae upon the addition of proteolytic enzymes that cleave proteins to generate carboxy terminal arginine peptides would support the hypothesis that pheromones are generated by enzymes acting upon the egg membrane proteins. The effects of trypsin were studied because it cleaves proteins and specifically generates carboxyl terminal arginine peptides.

A second objective was to test whether trypsin inhibitor also induces the female's larval release behavior. Even though they are ubiquitous and have evolved independently many times (Kassell, 1970; Lakowski and Kuto, 1980), trypsin inhibitors have poorly understood physiological functions (Lakowski and Kuto, 1980). We hypothesize that trypsin inhibitors, which are identified by their enzyme inhibitory properties, can also function as signal molecules.

Based upon the synthesis pathways of known polypeptide hormones, there are two alternative ways to generate proteinaceous signal molecules. First, a large polypeptide can be cleaved to generate specific peptides. Beta-lipoprotein, endorphins, and basopressins are examples of this mechanism. The alternative

is to synthesize a protein and then fold it to the active form. Prolactin, follicle-stimulating hormone, and growth hormone are examples of this mechanism. An advantage to the second method is the availability of the tertiary protein structure for contribution to the specificity of binding.

Proteinaceous trypsin inhibitors are another example of folded and constrained proteins. These inhibitors form 1 : 1 complexes with trypsin with high specificity and affinity. A constrained portion of the protein fits into the active site of trypsin, while additional parts of the molecule contribute to the high affinity of binding. The hypothesis in the present study is that trypsin inhibitors also can function as signal molecules. This hypothesis would be supported by demonstrating that the larval release behavior of *R. harrisii* is induced by trypsin inhibitors.

METHODS AND MATERIALS

Ovigerous *Rhithropanopeus harrisii* (Gould) were collected from the Neuse River estuary in North Carolina. Crabs were maintained on 10 ppt seawater at 25°C on a 14-hr light–10-hr dark cycle timed to the natural photoperiod. Even though responsiveness to pheromones does not vary rhythmically over the day (Forward and Lohmann, 1983), crabs were tested in the 6-hr interval in the middle of the light phase. Test solutions were made up immediately prior to use in 10 ppt seawater, which had been filtered to remove particles >0.45 μm. Test chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri).

There were two sets of experiments. First, the effects of bovine trypsin (T8253) and arginine (T0134, Sigma) on premature egg hatching were tested. Hatching time is highly predictable and premature hatching does not normally occur (Forward et al., 1982). The second set of experiments determined the concentrations of trypsin and trypsin inhibitors (T9003 soybean; A9024 Human αT; T4385 turkey; T2011 chicken; T0738 lima bean; all from Sigma) that induced the females' larval release behavior. In the first experiments the number of eggs that detached from the female's egg mass and the number that hatched prematurely were determined after incubation of ovigerous females for 4 hr in 40 ml of different trypsin concentrations. The crabs remained alive at all test concentrations and had egg masses of similar size. Embryo development of the test crabs was determined by egg yolk content and embryo eye size. Only crabs with eggs that would hatch within 36 hr were used experimentally. Incubation in trypsin occurred at a time in the light–dark cycle when eggs never hatch, i.e., the middle of the light phase (Forward et al., 1982). Ten crabs were tested at each concentration.

The bioassay for induction of the larval release behavior upon exposure to

the trypsin and trypsin inhibitors was frequency of abdomen pumping by ovigerous females (Forward et al., 1987). The general procedure was first to place a crab in a 7.0-cm-diameter finger bowl containing 40 ml of 0.45-μm filtered 10 ppt seawater. The number of times she pumped her abdomen in a 2-min interval established the unstimulated pumping rate. The crab was then transferred to a test solution (40 ml), and the number of pumps in a 2-min interval counted. The crab was considered to respond to the test solution if the number of pumps exceeded those in the initial seawater exposure. Crabs that responded usually pumped vigorously in the test solution, whereas upon initial exposure to seawater, pumping was infrequent. The control measured pumping in crabs transferred from seawater to seawater. Individuals were tested only once at each test concentration, presented in an ascending order. The minimum time between retesting of individuals was about 30 min. Crabs were maintained in 0.45-μm filtered seawater between tests, and solutions were remade after each 10 crabs tested. Pumping by females with immature embryos does not result in egg hatching or removal of the eggs from the egg mass. Short-term exposure (< 10 min) to trypsin or trypsin inhibitors in the pumping assays did not cause premature egg hatching or larval release.

A minimum of 30 crabs was tested at each concentration, and all crabs had eggs that would hatch within three days. Significant differences between controls and treatments were established by determining a Z statistic for testing differences between two proportions (Walpole, 1974).

RESULTS

Treatment with either porcine or bovine trypsin induced premature release of eggs and larvae of *R. harrisii* (Figure 1A and 2A). Premature detachment of eggs is normally rare in the laboratory and females never release larvae prematurely. Released larvae were immobile but alive, as indicated by heartbeat rates, which suggests premature hatching was enzymatic rather than because of larval movements. The lowest effective concentration of porcine trypsin was about 10^{-6} M, and both egg and larval release increased with concentration (Figure 1A). For bovine trypsin, prematurely released larvae were observed at the lowest test concentration (10^{-7} M), and a dramatic effect was evident at 10^{-5} M (Figure 2A).

Both porcine and bovine trypsin also induced the pumping response (Figure 1B and 2B). Threshold concentrations were below those for premature larval release: 10^{-7} for porcine trypsin and 10^{-8} M for bovine trypsin. The typical biphasic response pattern to pure neutral–basic peptides was observed (Forward et al., 1987). Responses to pure peptides generally increase to an optimum and then decrease to control levels with further increases in concentration. This

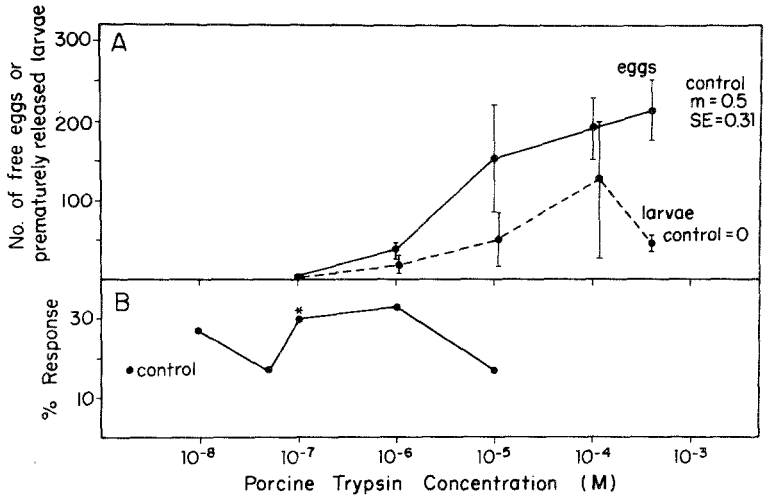

FIG. 1. The number of prematurely released eggs and larvae (A) and pumping response (B) on incubation of ovigerous females with late-stage embryos in different concentrations of porcine trypsin. Control indicates responses to filtered seawater alone. Means and standard errors are plotted in A. Asterisk (B) indicates the lowest concentration to induce responses that are significantly ($P < 0.05$) greater than control levels. Sample sizes in A and B were 10 and 30, respectively.

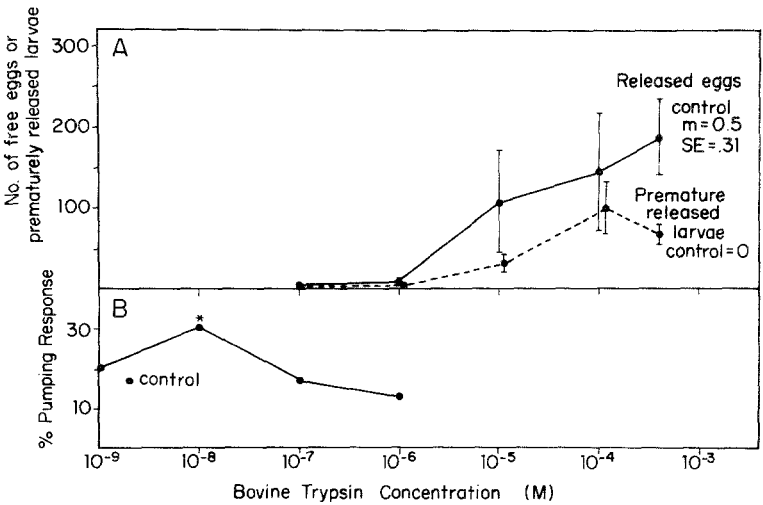

FIG. 2. The number of prematurely released eggs and larvae (A) and pumping response (B) on incubation in different concentrations of bovine trypsin. Control indicates responses in filtered seawater alone. Means and standard errors are plotted in A. Asterisk (B) indicates the lowest concentration to induce responses that are significantly ($P < 0.05$) greater than control levels. Sample sizes in A and B were 10 and 30, respectively.

suggests that trypsin evokes pumping through proteolytic generation of peptides.

Six trypsin inhibitors from three families (Lakowski and Kuto, 1980) were tested for their ability to induce the pumping response (Figure 3). All the inhibitors evoked the responses. They were more active than trypsin; thresholds ranged from 10^{-9} to 3×10^{-8} M (Table 1). As the threshold increased, the effective concentration range and maximum percent response declined (Table 1).

A final series of experiments tested pumping upon addition of each family of trypsin inhibitor combined with a threefold excess of porcine trypsin (Table 2). With the exception of chicken trypsin inhibitor, concentrations of inhibitors were those that evoked maximum percentage response (Figure 3, Table 1). The trypsin concentrations were at or below the threshold (10^{-7} M) for trypsin alone that induced pumping (Figure 1B). The combinations eliminated the pumping response to all five trypsin inhibitors (Table 2), which indicates the trypsin–trypsin inhibitor complexes are inactive as pheromone mimics. These results

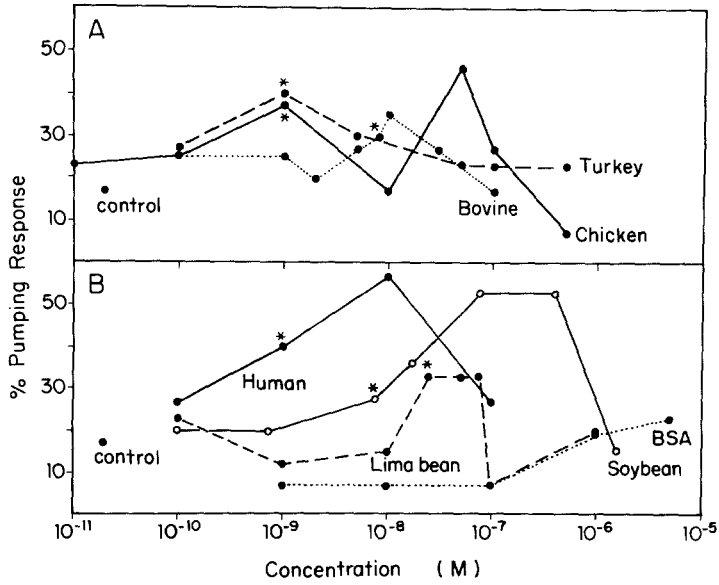

FIG. 3. The percentage of ovigerous crabs showing a pumping response upon exposure to different concentrations of bovine, chicken, turkey (A); lima bean, soybean, and human trypsin inhibitors and BSA (bovine serum albumin) (B). The sample size was 30, and the asterisks indicate the lowest concentration to evoke a response significantly ($P < 0.05$) greater than the control.

TABLE 1. RESPONSES TO TRYPSIN INHIBITORS[a]

Trypsin inhibitor range	Molecular weight[b]	Response threshold	Inhibitor family[c]	Effective conc.	Maximum percentage
Soybean	20,100	7.5×10^{-9} M	Kunitz	2.0 logs	50
Human	58,000	10.0^{-10} M	Kunitz	1.0 logs	57
Bovine	6,500	1.2×10^{-8} M	Kazal	0.25 logs	35
Chicken	29,000	10.0^{-9} M	Kazal	0.5 logs	46
Turkey	28,000	10.0^{-9} M	Kazal	1.0 logs	40
Lima bean	9,000	7.0×10^{-8} M	Bowman-Birk	0.5 logs	33

[a] Response threshold is the lowest concentration to evoke a pumping response that is significantly ($P < 0.05$) greater than control levels in filtered seawater alone. Effective concentration range indicates concentration range over which the trypsin inhibitor evokes a significant pumping response. The highest response level is shown as the maximum percentage response.
[b] Provided by Sigma Technical Services representative.
[c] Laskowski and Kuto (1980).

support the concept that pumping is induced by the catalytic site binding portion of the trypsin inhibitor and/or peptides generated by enzymatic activity of trypsin.

These results also indicate that pumping is not induced simply by the presence of protein. The trypsin–trypsin inhibitor complexes did not evoke the response. Similarly, bovine serum albumen failed to induce the response over a wide concentration range (Figure 3B).

TABLE 2. PERCENT PUMPING RESPONSE ON EXPOSURE TO TRYPSIN INHIBITOR COMBINED WITH PORCINE TRYPSIN[a]

Trypsin inhibitor	Trypsin inhibitor concentration (M)	Porcine trypsin concentration (M)	Pumping response (%)
Human	1.0×10^{-8}	3.0×10^{-8}	7
Turkey	1.0×10^{-9}	3.0×10^{-9}	20
Chicken	5.0×10^{-8}	3.0×10^{-7}	13
Bovine	1.0×10^{-8}	3.0×10^{-8}	27
Lima bean	5.0×10^{-8}	1.5×10^{-7}	17

[a] See Figure 3. Trypsin inhibitor was tested at concentrations that evoke the highest percent response (Figure 3) when tested alone. The control percent response was 17% and the sample size for inhibitor-trypsin combinations was 30. In no case is the percent response significantly higher than the control.

DISCUSSION

The basic features of the proposed model for egg hatching by the crab *Rhithropanopeus harrisii* specify that hatching is controlled by the developing embryos (Forward and Lohmann, 1983) and is communicated to the female by the release of peptide pheromones from the eggs (Rittschof et al., 1985). These pheromones induce the female to perform larval release behavior involving vigorous pumping of the abdomen. This mechanical shaking is the final act in the hatching process and serves to rupture fragile egg membranes.

A primary objective was to determine whether the peptide pheromones could be generated by proteolytic cleavage of egg membrane proteins. The external addition of low concentrations of either bovine or porcine trypsin rapidly induced the female's larval release behavior, presumably through degradation of the egg membrane proteins to bioactive peptides. With longer incubation at higher concentrations, both trypsins caused the detachment of eggs from the egg mass and premature hatching. These results support the concept that peptide pheromones are generated by enzymes acting on the egg membranes and that trypsinlike enzymes are responsible for degradation of the egg membranes at the time of hatching.

The second objective was further investigation of the peptide receptor by determining whether the female's larval release behavior could be induced by exposure to trypsin inhibitors. The six test trypsin inhibitors were from three independently evolved families (Table 1) (Lakowski and Kuto, 1980) of large (6500-to 58,000-dalton) protein trypsin inhibitors. The families have the ability to bind to the catalytic site of trypsin as their only common feature. Although there are sequence homologies that allow the inhibitors to be grouped into families, even within each family the most common feature is the ability to inhibit trypsin (see Laskowski and Kato, 1980). The fact that all these inhibitors evoke larval release behavior in the free state and do not evoke the response when complexed with trypsin suggests that it is the portion of the molecules that interacts with the trypsin catalytic site that induces pumping.

Knowledge of the native ligand in the crab system and demonstration of its specific origin are essential to verification of the model for egg hatching and larval release behavior (Forward et al., 1987). Knowledge of natural ligands and their origins is not necessary to support the concept that protease inhibitors can function as signal molecules or allomones. The pumping response was evoked by all trypsin inhibitors at very low concentrations. The active concentrations fell within the range of the native peptide pheromone (Rittschof et al., 1985; Forward et al., 1987). Responses to the trypsin inhibitors were specific for these molecules, because the crabs failed to respond to equivalent protein

concentrations of bovine serum albumin and trypsin–trypsin inhibitor complexes. These results provide evidence for the existence of external receptors for trypsin inhibitors. We suggest that similar types of receptors also may occur within other organisms. Receptors like those that detect the variety of carboxy terminal arginine regulatory peptides (Hruby and Rich, 1983) are likely candidates. If these receptors exist, then it is attractive to speculate that trypsin inhibitors may also function as hormones, hormone mimics, pheromones, and/or allomones.

There are many examples of high internal concentrations of trypsin inhibitors, such as those found in leguminous seeds and avian egg white (Kassell, 1970). Inhibitors at these locations (seeds, eggs, etc.) serve a defense function through enzyme inhibition (Vogel et al., 1968; Bishop et al., 1981; Neurath, 1984) or possibly by mimicking natural hormones or pheromones. These mimics could disrupt metabolism or behavior. A multifunctional role of proteinase inhibitors is supported by the debate that exists as to what are and are not protease inhibitors (Salvesen and Nagase, 1989). The data presented here support the idea also set forward by Salvesen and Nagase (1989) that definitions of function should be driven by practical considerations. A hormonal or signaling function would explain the widespread and often puzzling occurrence of "protease" inhibitors (Laskowski and Kato, 1980).

Trypsin inhibitors inactivate trypsinlike serine proteases by binding to the catalytic site. The present study indicates these inhibitors also bind to and excite receptors for the carboxy terminal arginine peptides generated by trypsinlike enzymes. This combination of effects is unusual because both enzyme inhibition and stimulation of the receptor require high specificity and binding affinity (Kassell, 1970; Forward et al., 1987). Thus the enzyme-active site and the receptor-active site must be very similar chemically. However, the sites differ in that the enzyme catalyzes cleavage of a peptide bond, has low affinity for peptides, and has high affinity for trypsin inhibitors, whereas the receptor has a high affinity for short carboxy terminal arginine peptides (Forward et al., 1987; Rittschof et al., 1989) as well as trypsin inhibitors.

A single amino acid change in the catalytic site of trypsin could result in the requisite affinity for peptides without altering inhibitor binding affinity. Substitution with a basic amino acid for the active site serine in the enzyme would eliminate catalytic activity (Young et al., 1978). At the slightly basic ($\simeq 8.3$) pH of seawater, a basic residue in this position would concomitantly increase binding strength for short peptides by providing a positive charge to interact with the negative charge associated with the carboxyl group of the terminal amino acid of the peptide. Because this is the cleavage site for the enzyme and not directly involved in binding, there would be no effect on the binding of the

inhibitor. A structure with these features has been postulated as the receptor for the trypsinlike protease-generated C3a anaphlaxotoxin peptide (Unson et al., 1984).

Molecular graphics were used to develop a receptor–peptide model similar to that proposed by Unson et al. (1984). Lysine was substituted for ser 195 in the catalytic site of bovine trypsin and Ile-gly-arg inserted as the active tripeptide. This tripeptide is the most active peptide pheromone mimic (threshold = 10^{-15} M) tested thus far in the crab system (Rittschof et al., 1989). With this change, the pocket of the original enzyme that confers specificity for lysine or arginine is unchanged (Young et al., 1978). The molecular graphics showed that the tripeptide is stabilized by three salt bridges and one hydrophobic binding region. The charged residues necessary for salt bridges to the amino terminal, carboxyl terminal, and guanidinium side chain in the tripeptide are all in the appropriate locations. Specifically, peptide binding is stabilized by salt bridges: (1) between the peptide amino terminal and the carboxylate group of Asp 189 via a water molecule (Young et al., 1978), (2) between the guanidinium of the carboxyl terminal Arg and Asp 194, and (3) between the carboxyl of the terminal Arg and the amino side chain of Lys 195. Finally, hydrophobic interaction with the side chain of Ile in the peptide is provided by the main chain portions of residues 214–216, similar to the hydrophobic stabilization of the aliphatic part of lysine in bovine basic protease inhibitor (Young et al., 1978). Thus, a peptide receptor binding site could evolve from a protease catalytic site through a single amino acid change.

The idea of evolutionary relations between a receptor and an enzyme in the pathway involved in the production of the signal molecule has been suggested for steroid hormone receptors (Baxter, 1983, in Norman and Litwack, 1987). The concept of the evolutionary change of proteases into molecules with new physiological functions is well established (Neurath, 1984). The spectrum of known evolutionary variants ranges from enzyme specificity and function (Marous et al., 1971; Davie and Fujikawa, 1977; Brodrick et al., 1978; Woodbury and Neurath, 1980; Muller-Esterl and Fritz, 1981; Reid and Porter, 1981) to molecules, such as streptokinase, which acts as a steric activator and has lost its catalytic activity because of an amino acid change in the active site (Magnusson et al., 1976; Sottrup-Jensen et al., 1978; Castellino, 1979; Jackson and Tang, 1982). Thus it is highly probable that a peptide receptor could evolve through minor alteration of a proteolytic enzyme.

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EFFECTS OF SOIL NITROGEN LEVEL ON FERULIC ACID INHIBITION OF CUCUMBER LEAF EXPANSION[1]

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Abstract—It has been suggested that the allelopathic activity of phenolic acids should be primarily important in soils of low fertility. If this is true, then plant growth inhibition by phenolic acids may be unimportant in managed agricultural soils. The objective of this study was to determine how soil nitrogen (N) level might modify phenolic acid inhibition of growth. Cucumber seedlings (*Cucumis sativus* cv Early Green Cluster) grown in containers in growth chambers under varying N levels (5, 10, 15, 20, and 25 μg N/g soil) in Portsmouth B_1-horizon soil material were treated with ferulic acid (0 or 10 μg/g soil). Nitrogen and ferulic acid (FA) were applied every other day to the soil surface. The amount of FA in the soil solution declined with depth in the containers. A more rapid disappearance of FA from the soil solution was observed for the last FA treatment (0% recovered after 10 hr on day 23) than the first treatment (44% recovered after 10 hr on day 13). Both low N (5 μg N/g soil) and FA treatments reduced shoot dry weight, the mean absolute (AGR) and the mean relative (RGR) rates of leaf expansion, and increased the root–shoot ratio. High N treatments reduced shoot dry weight and the AGR. Ferulic acid inhibited cucumber seedling growth over a range of N concentrations, suggesting that the allelopathic activity of phenolic acids may be important in both nutrient limiting and nonlimiting soils for some species.

Key Words—Allelopathy, ferulic acid, *Cucumis sativus*, leaf expansion, nitrogen levels, growth inhibition.

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INTRODUCTION

Phenolic acids have been identified among a variety of water-soluble allelopathic compounds from plants or plant debris. In free form and at an effective concentration in the soil, these compounds are thought to influence plant growth and distribution in natural ecosystems and crop productivity in agricultural systems (Rice, 1984). In addition to plant inputs, a variety of factors determine the state and concentrations of phenolic acids in soil. Phenolic acids are subject to microbial utilization, polymerization, adsorbtion to soil particles, and adsorbtion/absorbtion by seeds and roots (Martin et al., 1972; Turner and Rice, 1975; Haider and Martin, 1975; Dalton et al., 1983; Shann and Blum, 1987). Other factors, such as pH, temperature, moisture level, and soil organic matter, will also modify the activity and availability of these compounds (Harper and Balke, 1981; Dalton et al., 1983; Einhellig and Eckrich, 1984; Dalton, 1984; Blum et al., 1985a,b; 1987).

There are also reports that indicate soil fertility may affect the impact of phenolic acids on plant growth. Glass demonstrated that phenolic acids can decrease root uptake of both cations and anions through nonspecific effects on root cell membranes (Glass, 1973, 1974; Glass and Dunlop, 1974). This suggests that the inhibitory effects of phenolic acids on plants grown under low nutrient levels may be greater than on plants grown under adequate nutrient levels.

Stowe and Osborn (1980) grew barley (*Hordeum vulgare* L.) in a sand culture with varying levels of nitrogen and phosphorus and phenolic acid treatments. They found that the phenolic acids used were uniformly and significantly inhibitory only at low nutrient concentrations and concluded that allelopathy by means of phenolic compounds should be most important in soils of low fertility. If this is true, then plant growth inhibition by phenolic acids may be unimportant in managed agricultural soils. Hall et al. (1983) observed an elimination of chlorogenic acid inhibition of pigweed growth when nutrient solution was added to soil. Blum and Dalton (1985), however, found that cucumber plants grown in nutrient culture were equally inhibited by ferulic acid at both limiting and nonlimiting nutrient levels.

The objective of this study was to determine how soil nitrogen level might modify phenolic acid inhibition of plant growth. Possible treatment effects on dry weight, root distribution, and phenolic acid distribution and disappearance in the soil were also investigated. Ferulic acid (FA) was chosen as a representative phenolic acid because it is an allelopathic compound (Rice, 1984) that has been extracted from plants (Bates-Smith, 1956; Liebl and Worsham, 1983), and isolated from soil (Whitehead, 1964; Whitehead et al., 1981). Ferulic acid is known to be a product of lignin degradation (Turner and Rice, 1975; Martin and Haider, 1976). Cucumber seedlings (*Cucumis sativus* cv Early Green Clus-

ter) were used in these experiments because they are sensitive to FA, predictable in their behavior, and quick to respond to FA applications. There is also a linear correlation between the total surface area of the leaves and dry weight for cucumber seedlings; hence, data on growth can be obtained without destructive sampling (Blum and Dalton, 1985; Blum et al., 1985a,b).

Portsmouth B_1-horizon soil material was chosen as a growth medium because a considerable amount of information is known concerning the behavior of FA in this substrate under both sterile and nonsterile conditions (Dalton et al., 1983; Blum et al., 1987; Blum and Shafer, 1988).

METHODS AND MATERIALS

General Procedure. Cucumber seeds were seeded into trays containing vermiculite and Hoagland's solution and then placed into a growth chamber in the South Eastern Plant Environment Laboratory. The trays were covered with foil to exclude light until the seedlings emerged from the vermiculite. On day 3 or 4, the seedlings were exposed to light for 12 hr and then transplanted into styrofoam containers with 276 g of soil material. There were no drainage holes in the containers to ensure that all added nutrient solution and/or FA solution was available to interact with seedling roots. The growth medium consisted of a 2:1 mixture of washed river sand and Portsmouth B_1-horizon soil material (fine loamy, mixed, thermic Typic Umbraquualts). The Portsmouth soil (Blum et al., 1987) was obtained from the N.C.S.U. agricultural research farm in Plymouth, North Carolina. It was sieved, air-dried, and adjusted to pH 5.2 with calcium hydroxide. The sand was rinsed with deionized water and then air-dried. A layer of quartz gravel (60 g) was placed on the soil surface to minimize evaporation and algal growth.

The containers with seedlings were supplied every other day with various amounts of a modified Hoagland's solution adjusted to pH 5.5 (see later sections) and deionized water. Water was added as needed on alternate days to compensate for evapotranspiration. Sufficient nutrient solution and water was added each day to bring the weight of each container to 360 g (field capacity) at the beginning and 380 g towards the end of the experiments (initial weight of soil, gravel, and cup was 340 g). This increase in the amount of solution added to the containers was necessary to compensate for the increase in transpiration by the growing seedlings. The seedlings were placed on carts in a growth chamber, which was maintained at a 25–21 °C day–night temperature, 80% relative humidity, and a 12-hr photoperiod (photosynthetic photon flux density 647 μmol/m^2/sec). Each cart held a complete set of treatments. The carts were rotated daily to minimize position effects.

The lengths and widths of all true leaves on each seedling were recorded

on the days the seedlings received nutrient solution and/or FA solution, with measurements initiated when the first true leaf was at least 10 mm in length. These data were subsequently used to calculate the leaf area and the absolute and relative rates of leaf expansion (Radford, 1967). The seedlings were harvested on day 20 or 25 to determine dry weight of the roots and shoots. All data were analyzed using the Statistical Analysis Systems programs for analysis of variance and regressions (SAS Institute Inc., Cary, North Carolina). Least significant differences (LSD 0.05) are presented as a measure of experimental precision. Inferences are based on the appropriate analysis of variance or regression analysis.

Leaf Area Model. Seedlings were grown under a range of nutrient levels and harvested on day 20. The length and width of each leaf was recorded before the leaf area was determined with a Licor leaf area meter (LI-3000). These data were used to develop a regression model that could estimate leaf area from the length and width measurements. This eliminated the need for destructive sampling in all subsequent experiments.

Nutrient Level Determination. The objective of the first experiment was to determine an appropriate level of nutrient solution for seeding growth. Five different nutrient treatments (6.5, 8.0, 9.5. 11.0, 12.5 ml modified Hoagland's solution) and 16 replications of each treatment were used. The Hoagland's solution was quadruple strength and contained no micronutrients. Quadruple strength was used to reduce the volume of solution added to the containers every other day. Micronutrients were omitted since the soil contained adequate amounts and the addition of micronutrients resulted in toxicity symptoms (unpublished data).

Interaction Experiment. The objective of the second experiment was to determine if the effects of FA on cucumber seedling leaf expansion were modified by varying the soil nitrogen (N) level. Quadruple-strength Hoagland's solutions with five N levels (25, 20, 15, 10, and 5 μg N/g soil) were used. The lowest N level was chosen to allow for the development of N-limited growth during the experimental time period. The two sources of N in Hoagland's solution were $Ca(NO_3)_2 \cdot 4H_2O$ and KNO_3. To reduce the N level of a given solution, either $Ca(NO_3)_2 \cdot 4H_2O$ and/or KNO_3 was omitted and substituted with $CaCl_2$ and/or KCl. The seedlings received either no FA or 10 μg/g soil. One milliliter (2760 μg FA/ml) of FA solution was mixed with nutrient solution (8 ml) prior to treatment, and then water was added to bring the total volume applied to 10 ml. The same procedure was followed for the control N treatments except no FA was added. There were eight replications for each treatment. The seedlings were harvested on day 23. At harvest, the shoots were cut at soil level, the soil of each container was then cut transversely into three sections of equal height and the corresponding portions of the root system at each level (top,

middle and bottom) were obtained by gently rinsing soil from roots. Root dry weights were then determined for each level.

Soil Extractions. The objective of the third experiment was to determine the vertical distribution of FA in the soil container and the rate of disappearance of FA from the soil. One FA level (10 μg/g soil), three N levels (5, 15, and 25 μg N/g soil), four extraction times, and three replications were used. The first extraction was made immediately after the first FA application (day 13), the second 10 hr later, the third on day 23 immediately after the last FA application, and the fourth 10 hr later. Ferulic acid was added every other day. Prior to soil extraction, the shoots were cut off at soil level and then two transverse cuts were made through the soil producing three soil sections of equal height (top, middle, bottom). Once roots were removed, each soil section was mixed, and 20-g and 30-g samples were taken from each section. The 20-g samples were weighed before and after drying at 40°C to determine moisture content. The 30-g samples were added to Erlenmeyer flasks with 50 ml of deionized water and placed on a wrist action shaker for 5 min. A 1-ml subsample was taken from each flask and centrifuged for 5 min in a Fischer Micro-Centrifuge model 235B. The supernatant was then filter sterilized (0.2 μm) and injected into a Waters high-performance liquid chromatograph for quantification of FA (Blum and Dalton, 1985).

RESULTS

Leaf Area Model. The equation for the linear regression model that best described the relationship between length, width and area was:

$$\text{Area (cm}^2) = -2.206 + [0.009^* (L\ ^*W)]$$

where length (L) and width (W) are in mm.

The r^2 for this regression line was 0.98 ($P = 0.0001$, $N = 106$). This equation was used to calculate leaf area in all subsequent experiments.

Nutrient Level Determination. The results of the nutrient experiment (Table 1) indicate significant inhibition of the mean absolute rate of leaf expansion (AGR, cm^2/2 days) for the nutrient levels in excess of 9.5 ml during the first growth period (days 11–13) and the final two growths periods (days 17–19 and 19–21). The means for the other growth periods (days 13–15 and 15–17) were not significantly different. The 6.5, 8.0, and 9.5 ml nutrient solution treatments encompassed the range for best growth in the chamber. Eight milliliters of quadruple-strength Hoagland's solution was chosen for all subsequent experiments.

Interaction Experiment. There was no significant N effect on the AGR during growth period 11–13 (Table 2 and Figure 1A). During the 13- to 15-day

TABLE 1. MEAN ABSOLUTE RATES OF LEAF EXPANSION OF SEEDLINGS RECEIVING DIFFERENT AMOUNTS OF QUADRUPLE-STRENGTH HOAGLAND'S SOLUTION EVERY OTHER DAY

Nutrient solution (ml)	Mean absolute growth rate (cm^2/2 days) for growth period (days)				
	11–13	13–15	15–17	17–19	19–21
6.5	9.63	6.31	20.31	18.89	37.68
8.0	9.01	5.74	19.16	19.43	40.20
9.5	8.44	6.04	21.10	18.53	40.47
11.0	7.70	5.42	18.54	16.56	33.93
12.5	7.57	5.20	17.16	15.45	30.91
$LSD_{0.05}$	1.03	NS	NS	1.63	3.72

growth period, however, the AGR of the lowest N treatment (5 μg N/g soil) was significantly larger than that of the 15-, 20-, and 25-μg N treatments, and during the 15- to 17-day growth period, the AGR of both the 5- and 10-μg N treatments were significantly larger than all others. A reduction in the AGR of

TABLE 2. MEAN SQUARES FROM ANOVA TESTING FOR NITROGEN (N, $df = 4$), FERULIC ACID (FA, $df = 1$) AND INTERACTION (N*FA, $df = 4$) EFFECTS[a]

	N	FA	N*FA	Error	*df* Error
Shoot	0.01081*	0.20352*	0.00359	0.00199	66
Total root	0.00746	0.00818	0.00606	0.00400	66
Root-shoot	0.08414*	0.13608*	0.03494	0.03298	66
Final leaf area	1384.30*	30098.6*	231.203	164.023	70
AGR					
Days 11–13	5.13000	0.20550	1.32000	1.65000	70
Days 13–15	7.89000*	26.0400*	5.80000	2.55000	70
Days 15–17	38.6300*	508.510*	14.1900	7.94000	70
Days 17–19	106.460*	1859.46*	32.4700	23.7800	70
Days 19–21	182.140*	2000.37*	54.6100*	16.2800	70
Days 21–23	386.098*	3137.28*	81.0100*	21.0000	70
RGR					
Days 11–13	0.04400	0.02514	0.01309	0.05857	70
Days 13–15	0.00717	0.05247*	0.02120	0.01064	70
Days 15–17	0.01550	0.49361*	0.00493	0.00645	70
Days 17–19	0.02251	0.38290*	0.01160	0.01186	70
Days 19–21	0.06090*	0.01120	0.02583*	0.00845	70
Days 21–23	0.02537*	0.00743	0.00350	0.00212	70

[a] Significant (0.05) effects are indicated by an asterisk.

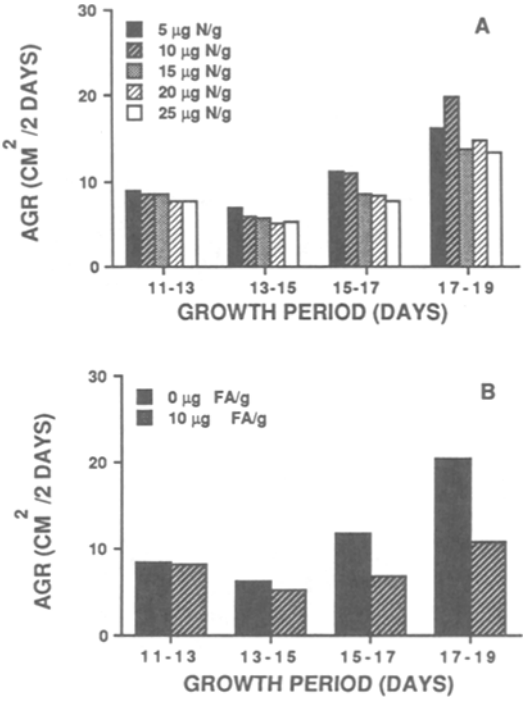

FIG. 1. (A) Mean absolute (AGR) rates of leaf expansion of cucumber seedlings receiving different amounts of nitrate nitrogen (5–25 μg N/g soil). (B) AGR of cucumber seedlings received either 0 or 10 μg ferulic acid (FA) per gram of soil.

the 5-μg N treatments was noted during the 17- to 19-day growth period, indicating that N had become limiting for growth.

FA treatments reduced the AGR 18%, 42%, and 47%, during the second (days 13–15), third (15–17), and fourth (17–19) growth periods, respectively (Figure 1B).

There was a significant interaction between N and FA on the AGR during the 19- to 21- and 21- to 23-day growth periods (Figure 2A and B). FA suppression of the AGR during these final two growth periods was similar for the 10- to 25-μg N (31–41% inhibition) treatments, but reduced in the 5-μg N treatments (17%).

There was a significant FA inhibition of the mean relative rates of leaf expansion (RGR, $cm^2/cm^2/2$ days) during growth periods 13–15, 15–17, and 17–19, but no significant FA effects were observed during the first or final growth period (Table 2, Figure 3A). A significant interaction between N and

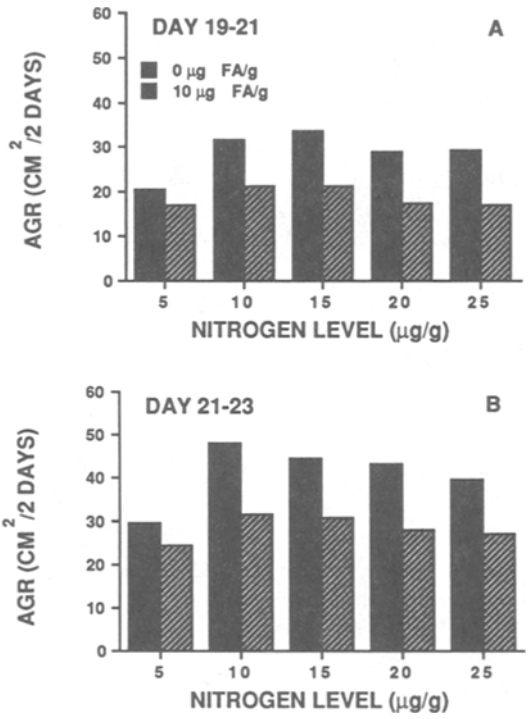

FIG. 2. Mean absolute rates of leaf expansion (AGR) of cucumber seedlings receiving either 0 or 10 μg/g ferulic acid (FA) and different amounts of nitrate nitrogen (5–25 μg N/g soil) for (A) growth period (days) 19–21, and (B) growth period 21–23.

FA on the RGR occurred during the 19- to 21-day growth period (Figure 3B). During this growth period, the RGR of the control seedlings for the 5-μg N treatments was significantly lower than the RGR of the FA treated seedlings at the same N level. This contrasts with the other N levels, where the RGR of the control seedlings was either significantly greater or not significantly different than the FA treated seedlings. During the final growth period (Figure 3A and C), the only significant N effects noted were on the RGR of the 5-μg N treatments.

Both FA and N significantly affected final leaf area, shoot dry weight, and the root–shoot ratio (Tables 2 and 3). Maximum mean final leaf area and shoot dry weight was achieved in the 10-μg N treatment. FA reduced final leaf area by 32% and shoot dry weight by 25%. There was a significant FA and N effect on the root–shoot ratio (R/S) but not total root dry weight or distribution (mean root dry weights were 0.13, 0.03, and 0.03 for the top, middle, and bottom

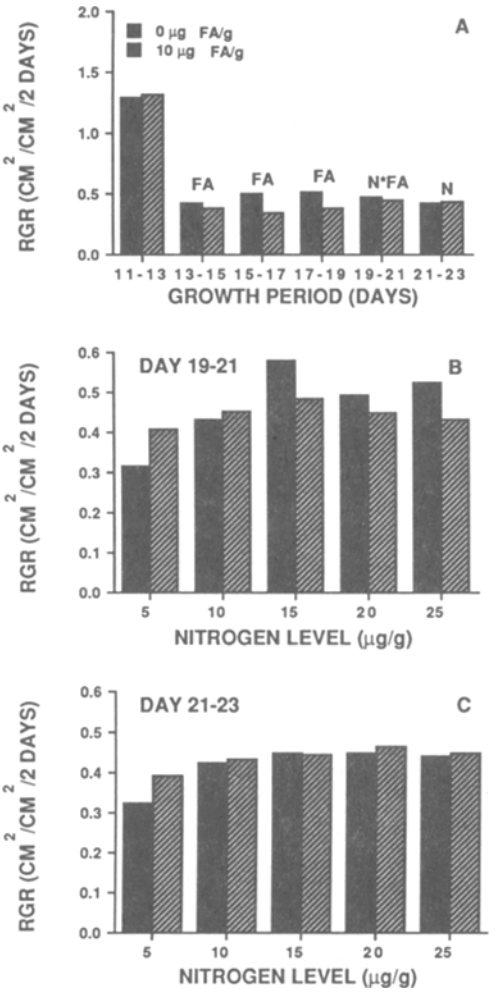

FIG. 3. (A) Mean relative rates of leaf expansion (RGR) of cucumber seedlings receiving either 0 or 10 μg ferulic acid/g soil. Significant effects are indicated by FA (ferulic acid), N (nitrogen), and N* FA (interaction). RGR of cucumber seedlings receiving either 0 or 10 μg FA/g soil and different amounts of nitrate nitrogen (5–25 μg/g soil) for (B) growth period (days) 19–21 and (C) growth period 21–23.

sections, respectively). The increase in the R/S observed for the FA and low N treatments were due, therefore, to a reduction in shoot dry weight. Maximum R/S values occurred in the 5-μg N treatments (Table 3). FA increased the final R/S ratio by 16%.

TABLE 3. NITROGEN AND FERULIC ACID EFFECTS ON MEAN FINAL LEAF AREA, SHOOT DRY WEIGHT, AND ROOT–SHOOT RATIO OF CUCUMBER SEEDLINGS

	Final leaf area (cm^2)	Final shoot dry wt. (g)	Root–shoot ratio
Nitrogen level ($\mu g/g$)			
5	92.42	0.33	0.65
10	114.99	0.41	0.53
15	104.71	0.38	0.51
20	97.78	0.38	0.48
25	93.72	0.37	0.46
$LSD_{0.05}$	9.06	0.03	0.14
Ferulic acid level ($\mu g/g$)			
0	120.12	0.43	0.48
10	81.33	0.32	0.57
$LSD_{0.05}$	5.73	0.02	0.08

Soil Extractions. Maximum recovery of FA occurred in the top soil section and declined with depth (Table 4). On day 13 the average recovery in all treatments immediately after FA application was 55% of the total amount (2760 μg) added, and 46% of this was extracted from the top soil section, 9% from the middle, and 0% from the bottom soil section (Table 4). A similar pattern was found immediately after FA application on day 23, where, on average, 64% of the total FA added was recovered (57% top section, 7% middle, and $<1\%$

TABLE 4. AVERAGE PERCENT RECOVERY OF FERULIC ACID (FA) AT DIFFERENT SOIL DEPTHS IMMEDIATELY AFTER APPLICATION (TIME = 0) AND 10 HR AFTER APPLICATION (TIME = 10) ON FIRST (DAY 13) AND LAST (DAY 23) TREATMENT DAYS[a]

Soil section	Recovery (%) day 13		Recovery (%) day 23		
0.05	Time = 0	Time = 10	Time = 0	Time = 10	LSD
Top	45.9	37.4	57.0	0.0	7.6
Middle	9.2	6.3	6.7	0.0	3.6
Bottom	0.0	0.1	0.5	0.0	NS

[a] The amount of FA applied to the soil surface was 2760 μg (10 μg/g soil). The height of each soil section was 2.3 cm. Mean soil dry weights of the top, middle, and bottom sections were 130, 90, and 56 g, respectively.

bottom section). The circumference of the styrofoam containers declined with depth, and so the soil weight and volume of the three sections declined accordingly. The top, middle, and bottom sections contained approximately 130, 90, and 56 g of dry soil, respectively. The average recovery of FA (in μg FA/g soil dry weight) in the top, middle, and bottom sections was 9.7, 2.8, and 0 on day 13 (time = 0) and 12.1, 2.0, and <1.0 on day 23 (time = 0). The mean total recovery of FA 10 hr after addition was 44% on day 13 and 0% on day 23.

DISCUSSION

FA at 10 μg/g soil was added to the soil surface, resulting in a FA distribution that varied with depth. The reduced recovery of FA immediately after treatment was due most likely to soil fixation and sampling procedures. The difference in recovery 10 hr after treatment between day 13 and 23 (44% vs 0%) was due most likely to accelerated soil microbial activity and/or uptake by larger root systems on day 23. Blum and Shafer (1988) noted that treatment of Portsmouth B_1-horizon soil material with FA resulted in the induction or selection of microorganisms that could utilize FA as a sole carbon source. In nutrient culture, uptake of FA by cucumber seedlings increased with the increase in size of the root system (Shann and Blum, 1987).

Root distribution also varied with depth. At harvest, an average of 66% of the root system was located in the top soil section and 17% in both the middle and bottom sections. There was no N or FA treatment effect on total root dry weight at final harvest. The observed root and FA distribution patterns clearly indicate that the majority of the FA and root interaction occurred in the top soil section. Whether seedling roots not in contact with FA compensated (i.e., exploited resources more efficiently) for roots in contact with FA is not known. Several authors, however, have suggested that the proportion of a plant's root system in contact with allelochemicals could significantly affect plant response (Patrick et al., 1964; Lynch, 1985; Blum and Rebbeck, 1988). Klein and Blum (1990) demonstrated in split-root experiments that cucumber seedling growth was inversely related to the proportion of the root system in contact with FA solution.

These data indicate that the N requirements of the growing seedlings changed with time and that during the final three growth periods the range of N concentrations included both limiting and inhibitory N levels. The pattern of RGR inhibition and recovery coincided with the observed increase and decline of FA inhibition of the AGR during the corresponding growth periods. The RGR of the 5-μg N treatments that received FA was actually higher than the control seedlings during growth period 19–21, and this accounts for the significant interaction. Clearly N was limiting to both control and treated seedlings

during this growth period, but the control seedlings were larger than the FA-treated seedlings and thus may have had higher N requirements for a given rate of growth. Seedling size differences were a result of the cumulative effects of the multiple FA treatments on the growth rates of treated seedlings. It is probable that this growth suppression of the treated seedlings was due largely to FA suppression of seedling water utilization (Blum and Dalton, 1985; Blum et al., 1985a) and not nutrient uptake, because strong treatment effects were observed in the presence of adequate N levels for growth.

FA inhibition of seedling growth for the fifth and sixth growth periods was lower than the fourth (days 17–19). This decline in growth inhibition may have been due to acclimation and/or microbial activity in the soil (Vaughn et al., 1983). Blum and Dalton (1985) found no clear evidence of acclimation of cucumber seedlings grown in nutrient culture. Blum and Shafer (1988), however, noted that multiple additions of FA to Portsmouth B_1-horizon soil caused shifts in the microbial community in this soil such that there was a significant increase in populations of phenolic-acid-metabolizing organisms. The more rapid decline of FA from the soil solution in this experiment would tend to support their observations, although other interpretations are possible.

In summary, FA inhibited cucumber seedling growth over a range of N concentrations including both limiting and inhibitory levels. This suggests that the allelopathic activity of phenolic acids may be important in both fertile and nutrient limited soils for some species.

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CONVERSION OF VERBENOLS TO VERBENONE BY YEASTS ISOLATED FROM *Dendroctonus ponderosae* (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—A variety of symbionts associated with bark beetles are capable of producing compounds that are used as pheromones by their hosts. We report that two yeasts associated with *Dendroctonus ponderosae* Hopkins, *Hansenula capsulata* Wickerham, and *Pichia pinus* (Holst) Phaff, are capable of converting *cis*- and *trans*-verbenol efficiently into verbenone. *trans*-Verbenol, which is produced by female *D. ponderosae*, acts as an aggregation pheromone for this scolytid, while verbenone, which other studies have indicated that microbe-reduced *D. ponderosae* are incapable of producing, acts as an antiaggregation pheromone. *D. ponderosae* appears to rely primarily on microbial symbionts for terminating aggregation and mass attack on individual host trees.

Key Words—*Dendroctonus ponderosae*, Coleoptera, Scolytidae, *Hansenula capsulata*, *Pichia pinus*, pheromones, *trans*-verbenol, verbenone.

INTRODUCTION

The hypothesis that microorganisms are involved in the production or modification of bark beetle pheromones has been debated for over half a century. Person (1931) hypothesized that the western pine beetle, *Dendroctonus brevicomis* Leconte, introduced a yeast into the inner bark of host trees that produced

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fermentation products responsible for secondary attraction. Callaham and Shifrine (1960) reported that yeast-inoculated phloem was attractive to various species of *Dendroctonus*.

More recently it has been confirmed that a variety of symbionts associated with bark beetles and other coleoptera are capable of producing compounds that are used as pheromones by their hosts. Bacteria isolated from female grass grub beetles, *Costelytra zealandica* (White), produced an unidentified compound that attracted male *C. zealandica* under field conditions (Hoyt et al., 1971). A *Serratia* species isolated from the bark beetle, *Phloeosinus armathus*, converted sabinene into terpinene-4-ol and α-terpineol, both of which were attractive to male and female *P. armathus* (Chararas et al., 1980). A strain of *Bacillus cereus* Frankland and Frankland, which was isolated from the guts of *Ips paraconfusus* Lanier, converted α-pinene to the host's pheromone *cis*-verbenol (Brand et al., 1975). Brand et al. (1976) demonstrated that fungi from the mycangia of *Dendroctonus frontalis* Zimmerman were capable of oxidizing *trans*-verbenol to the antiaggregation pheromone verbenone. The yeasts *Hansenula holstii* Wickerham and *Pichia pinus* (Holst) Phaff, also isolated from *D. frontalis*, produced metabolites that enhanced attraction of these beetles to a 1:1:12 mixture of frontalin–*trans*-verbenol–turpentine (Brand et al., 1977). French et al. (1984) reported that flying *Scolytus multistriatus* (Marsham) were attracted to agar cultures of *Bacillus subtilis* (Ehrenberg) Cohn, *Bacillus pumilis* Meyer and Gottheil, and *Enterobacter cloacae* (Jordan) Hormaeche and Edwards, isolated from elm trees. Several species of yeasts isolated from *Ips typographus* (L.) were capable of interconverting *trans*- and *cis*-verbenol and verbenone (Leufvén et al., 1984), of which the latter two function as pheromones for the host insects. These same yeasts were capable of producing a variety of oxygenated monoterpenes when grown in a phloem medium (Leufvén et al., 1988). Other more general examples of fungi and bacteria that are capable of oxidizing α-pinene and other monoterpenes are found in Bhattacharyya et al. (1960), Prema and Bhattacharyya (1962), Shukla et al. (1968), Fonken and Johnson (1972), and Keislich (1976).

Individual monoterpenes or extracts of resin have been found to be toxic to microorganisms associated with bark beetles, such as several *Ceratocystis* species (Cobb et al., 1968; DeGroot, 1972), *Trichosporium symbioticum* Wright (Raffa et al., 1985), and fungal symbionts of *Dendroctonus ponderosae* Hopkins (Shrimpton and Whitney, 1968), as well as being toxic to *Dendroctonus* species (Smith, 1965; Reid and Gates, 1970; Coyne and Lott, 1976; Raffa and Berryman, 1983b). Therefore, it is of adaptive advantage for the microorganisms to be capable of allylic oxidations of monoterpenes to detoxify these compounds and secondarily to use them as energy sources.

Our objective was to determine whether microorganisms associated with

the mountain pine beetle, *D. ponderosae*, were capable of the production or interconversion of pheromones of their host. We report the results of a study conducted on two yeasts associated with *D. ponderosae*, *Hansenula capsulata* Wickerham and *Pichia pinus*.

METHODS AND MATERIALS

Microorganisms. Cultures of two species of yeasts that are frequently associated with *D. ponderosae* were obtained from H.S. Whitney (Pacific Forestry Centre, 506 West Burnside Road, Victoria, B.C. V8Z 1M5, Canada), and were maintained on Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, Michigan). These two species were identified by L.J. Wickerham (Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois; now retired) as *Pichia pinus* and *Hansenula capsulata*.

Yeast Conversion Experiments. Conversion experiments were conducted using a procedure similar to that employed by Leufvén et al. (1984). α-Pinene, *trans*-verbenol, *cis*-verbenol, and verbenone were dissolved individually at 3 mg/ml in 95% ethanol; 250 μl of one of the ethanolic solutions was added to 250 ml Erlenmeyer flasks containing 50 ml of Sabouraud dextrose broth that had just been inoculated by aseptically transferring a small amount of cells from a fresh SDA culture of one of the yeasts. After 24 hr at 21–23°C with slight shaking, another 250 μl of the ethanolic solution was added, yielding a final ethanol concentration in the medium of 1% (v/v). All the flasks that contained broth that had been inoculated with one of the yeasts appeared cloudy after 24 hr, indicating growth of the yeasts. After a further 24 hr of incubation, 5 ml of the medium was extracted three times with 1 ml each of distilled pentane. Each treatment for each yeast was replicated three times, as were control treatments in which a sterile inoculating loop was dipped into the medium.

The *trans*-verbenol used in this study, which was 75% (−)-25% (+) and was contaminated with approximately 12% *cis*-verbenol and 0.8% verbenone, was obtained from Phero Tech Inc. (Vancouver, B.C.). Racemic *cis*-verbenol, which was contaminated with approximately 14% *trans*-verbenol, was obtained from Borregaard, A.S. (Sarpsborg, Norway), and racemic α-pinene (>99% pure) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Racemic verbenone (97% pure, as determined by gas chromatography) was obtained from Indukern (Barcelona, Spain).

Gas Chromatographic Analyses. Pentane extracts of yeast cultures were analyzed on a Hewlett Packard 5880A gas chromatograph equipped with a capillary inlet system and a flame ionization detector. A glass capillary column (30 m × 0.66 mm ID) coated with SP-1000 (Supelco, Inc., Bellefonte, Pennsyl-

vania) was used with the following temperature program: 120°C for 2 min, then increased by 4°C/min to 180°C. The injection port temperature was 260°C, the flame ionization detector temperature was 275°C, and helium was used as the carrier gas.

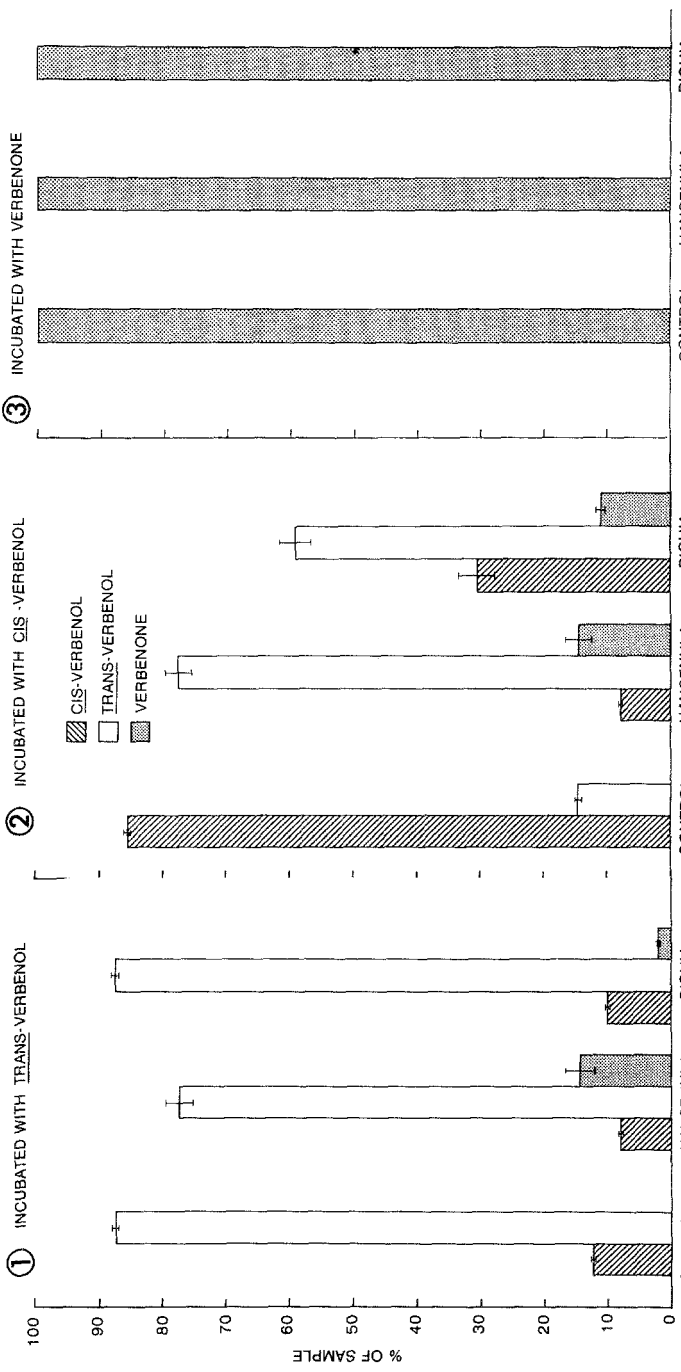
Each day that samples were analyzed by gas chromatography, a standard sample made up of α-pinene, *cis*- and *trans*-verbenol, and verbenone was also analyzed to aid in the identification of unknown compounds in the samples by comparison of retention times. In addition, these compounds were added periodically to an extract and that mixture was analyzed to ensure correct identification by cochromatography with unknown compounds in the extract. Selected extracts were analyzed using gas chromatography–mass spectroscopy to ensure proper identification of compounds. Amounts of detected compounds in yeast extracts were evaluated by comparing the relative proportions of the gas chromatographic peak areas (Leufvén et al., 1984). The sum of the gas chromatographic peak areas of *trans*- and *cis*-verbenol and verbenone was approximately the same after all incubations, indicating that the compounds were primarily interconverted, and not metabolized into other compounds.

RESULTS

Incubation of either yeast with *trans*-verbenol resulted in accumulations of verbenone, while the *trans*-verbenol control flasks, which were incubated without yeasts, contained no more verbenone than the 0.8% in the starting material (Figure 1). Incubation of *H. capsulata* with *trans*-verbenol caused an accumulation of 14.5 ± 2.1% verbenone, while incubation with *P. pinus* caused an accumulation of 2.2 ± 0.1% verbenone (Figure 1). For *H. capsulata* the decline in the relative amount of *trans*-verbenol, compared to that in the control flasks, appeared to indicate that the verbenone resulted from conversion of *trans*- as opposed to *cis*-verbenol.

Incubation of either yeast with *cis*-verbenol resulted in accumulations of *trans*-verbenol and verbenone, while the *cis*-verbenol control flasks, which were incubated without yeasts, contained both verbenols as well as verbenone at the original levels (Figure 2). Neither of the yeasts produced *trans*- or *cis*-verbenol from verbenone (Figure 3).

Extracts of α-pinene incubations did not contain detectable levels of α-pinene or terpene alcohols, even when the medium was extracted immediately following the addition of α-pinene. Thus the α-pinene was apparently bound very rapidly to an unidentified constituent of the medium, or it reacted to form a product that we did not detect through gas chromatographic analysis.



FIGS. 1–3. Mean percent content (±SE) of *trans*- and *cis*-verbenol and verbenone in cultures of *H. capsulata* or *P. pinus* incubated with *trans*-verbenol (Figure 1), *cis*-verbenol (Figure 2), or verbenone (Figure 3) for 48 hr. $N = 3$ for each treatment in each experiment.

DISCUSSION

The aggregation of *D. ponderosae* on host trees is due partially to the beetle-produced terpene alcohol *trans*-verbenol, in combination with host tree monoterpenes (Pitman et al., 1968), although low concentrations of the male-produced, multifunctional pheromones *exo*-brevicomin and frontalin may also be involved (Rudinsky et al., 1974a; McKnight, 1979; Conn et al., 1983; Borden et al., 1983, 1987; Chatelain and Schenk, 1984). Once a certain attack density is reached, the attack switches to adjacent trees (McCambridge, 1967; Geiszler and Gara, 1978; Geiszler et al., 1980), preventing the increased competition within hosts and reduced brood survival that occur at overly high attack densities (Reid, 1963). This switching appears to be due to a number of factors, including a decrease in resin flow (Renwick and Vité, 1970) and *trans*-verbenol production (Renwick and Vité, 1969; Borden et al., 1987) and the production of antiaggregation pheromones (Rudinsky et al., 1974a). Although several compounds produced by *D. ponderosae* have proven to be inhibitory to these beetles in laboratory and field tests (Ryker and Libbey, 1982; Ryker and Rudinsky, 1982; Libbey et al., 1985; Hunt and Borden, 1988), leading to speculation that they may act as antiaggregation pheromones, the antiaggregative activity of verbenone (Ryker and Yandell, 1983) is generally thought to be the most significant.

In lodgepole pine trees attacked by *D. ponderosae*, aggregation generally peaks on approximately the second day of attack, and then declines to zero within four to seven days of the initiation of attack (Raffa and Berryman, 1983a). The conversion of *trans*-verbenol into verbenone (Figure 1), particularly by *H. capsulata*, suggests that this termination of aggregation on trees attacked by *D. ponderosae* is the result of verbenone production by yeasts introduced into the trees by the attacking beetles. It is our hypothesis that the yeasts that are introduced into the attacked trees by *D. ponderosae* require a few days to reach a population size in the galleries large enough to enable them to convert significant quantities of *trans*-verbenol, which has been produced in large quantities by the beetles for the first few days, into verbenone. The switching of the attack to adjacent trees (McCambridge, 1967; Geiszler and Gara, 1978; Geiszler et al., 1980) thus would be due largely to the production of verbenone by yeasts (Figures 1 and 2).

The hypothesis that fungi introduced into attacked trees by bark beetles are responsible for the termination of aggregation has been proposed for other species. Brand et al. (1976) hypothesized that fungi introduced into the galleries of *D. frontalis* may convert *trans*-verbenol, which is involved in the aggregation of this bark beetle (Renwick and Vité, 1969, Payne et al., 1978), into verbenone, which can function as an antiaggregation pheromone (Rudinsky, 1973; Rudinsky et al., 1974a). Also, Leufvén et al. (1984) hypothesized that yeasts

introduced into the galleries of *I. typographus* may convert the aggregation pheromone *cis*-verbenol (Bakke et al., 1977) into verbenone, an antiaggregation pheromone (Bakke, 1981).

It is unclear why α-pinene was not extractable with pentane, which is an efficient solvent for neat α-pinene. It appears that the α-pinene was bound in some way within the water phase, so it may not have been available to be metabolized by the yeasts, or extracted. Alternatively, the α-pinene may have reacted to form a product that we did not detect through gas chromatographic analysis. Additional research is required to establish the capacity of these yeasts to metabolize α-pinene.

Although under laboratory conditions many microorganisms are capable of producing or interconverting compounds used as pheromones by bark beetles (Bhattacharyya et al., 1960; Prema and Bhattacharyya, 1962; Fonken and Johnson, 1972), and some of these microorganisms are found in association with bark beetles (Brand et al., 1975, 1976, 1977; Chararas et al., 1980; Leufvén et al., 1984), there is little evidence to suggest that these conversions are of any significance to the chemical ecology of bark beetles under natural conditions. However, several pertinent observations support the hypothesis that conversion of *trans*-verbenol into verbenone by symbiotic yeasts is of genuine significance to the chemical ecology of *D. ponderosae*.

First, although certain microorganisms such as *B. cereus* (Brand et al., 1975), and *Aspergillus niger* van Tieghem (Bhattacharyya et al., 1960; Prema and Bhattacharyya, 1962) are capable of the production of verbenols, it has been established that *D. ponderosae* that are free of these readily culturable microorganisms are capable of converting α-pinene vapors into *trans*-verbenol at levels equal to or above those found in wild beetles (Hunt and Borden, 1989). In contrast, axenically reared *D. ponderosae* did not produce quantifiable levels of verbenone, while beetles with their normal complement of microorganisms, which were fed on *P. contorta*, did (Hunt and Borden, 1989), as did yeasts associated with *D. ponderosae* (Figures 1 and 2). The production of verbenone by wild *D. ponderosae* from ingested α-pinene and the inability of these wild beetles to produce verbenone from α-pinene vapors (Hunt and Borden, 1989) suggest that the conversion is done by microorganisms in their guts. Verbenone is apparently not metabolized further by *H. capsulata* or *P. pinus* (Figure 3), so it would probably build up at significant levels in attacked trees. Thus it would be of adaptive advantage for individual *D. ponderosae* to exploit verbenone as a signal of an established attack in host trees that offer limited resources. *Candida nitratophila*, the only yeast isolated by Leufvén et al. (1984) that converted *trans*-verbenol into verbenone, has also been isolated from *D. ponderosae* (Shifrine and Phaff, 1956). There is a highly persistent association between *D. ponderosae* and its associated yeasts, suggesting a mutualistic association (Whitney, 1971). *H. capsulata* and *P. pinus* were associated closely

with *D. ponderosae* during brood development in lodgepole pine, and in an extensive survey no populations were found to be free of these yeasts (Whitney, 1971). Farmer (1965) also found that these yeasts were closely associated with *D. ponderosae* in lodgepole pine. Finally, these yeasts were isolated frequently from the maxillary mycangium of *D. ponderosae* (Whitney and Farris, 1970), compelling evidence of the importance of this symbiotic relationship.

When yeasts associated with *I. typographus* were quantified, it was found that those that can convert the aggregation pheromone *cis*-verbenol into the antiaggregation pheromone verbenone (Leufvén et al., 1984) were most prevalent on the beetles and in the galleries during those phases of the attack at which verbenone is produced in much higher levels than *cis*-verbenol (Leufvén and Nehls, 1986). During early attack phases, when aggregation is occurring on the trees and the beetles are producing large quantities of *cis*-verbenol, these yeasts were present on the insects in very low numbers (Leufvén and Nehls, 1986). We hypothesize that a similar temporal relationship exists between gallery development in *D. ponderosae* and the population of yeasts therein.

H. capsulata, *P. pinus*, and other yeasts capable of converting verbenols to verbenone (Leufvén et al., 1984), have been isolated frequently from *Dendroctonus* and *Ips* species (Callaham and Shifrine, 1960; Shifrine and Phaff, 1956). Many of these bark beetles use *cis*- or *trans*-verbenol as an aggregation pheromone and verbenone as an antiaggregation pheromone, suggesting that microbial involvement in the termination of bark beetle aggregation may be widespread. Of particular interest are insects such as *D. pseudotsugae* and *D. frontalis*, for which verbenone is a multifunctional pheromone, attractive at low concentrations and inhibitory at high concentrations (Rudinsky, 1973; Rudinsky et al., 1974a,b). For these species the low population levels of yeasts present in beetle galleries soon after the initiation of attack could contribute toward beetle aggregation by producing low levels of verbenone. In more advanced stages of attack the higher levels of verbenone produced by larger yeast populations would contribute toward terminating the attack on individual trees and directing it toward other trees.

It was our original intention to culture microorganisms from large numbers of *D. ponderosae* and to examine the abilities of the isolated microorganisms to produce and interconvert compounds that function as semiochemicals for their hosts. We now believe that such a survey would add little to what is already known about microbial involvement in bark beetle pheromone production and regulation. Considering the number of microorganisms that already have been found to be capable of producing or interconverting bark beetle pheromones in laboratory experiments, it is evident that the metabolic capacities for these conversions are common. It would be much more edifying to establish whether these metabolic capacities are relevant in nature. This question could be addressed by quantifying the production of metabolites by microorganisms

exposed to realistic levels of precursors under natural conditions and at population levels similar to those found naturally in association with bark beetles.

For the production of α-pinene-derived pheromones in *D. ponderosae*, it is now possible to refine and expand the model proposed by Borden (1984). The new model (Figure 4) proposes that most of the aggregation pheromone *trans*-verbenol formed in trees attacked by *D. ponderosae* is produced from inhaled α-pinene by the beetles' own enzymes (Conn et al., 1984; Hunt and Smirle, 1988; Hunt and Borden, 1989). The conversion of ingested α-pinene by microorganisms in the beetles' guts (Hunt and Borden, 1989), as well as autoxidation of α-pinene (Hunt et al., 1989), contribute in a minor way towards *trans*-verbenol production. *trans*-Verbenol also may be formed by microorganisms present in the beetles' galleries, although there are currently no data to support this possibility, and, therefore, this route is excluded from the model.

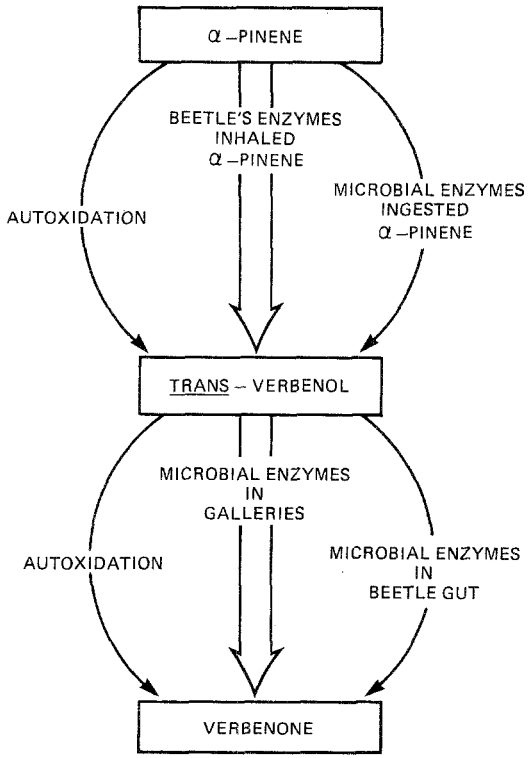


FIG. 4. Pathways for the production of α-pinene-derived pheromones in *D. ponderosae*. Wide arrows down the middle of the model denote major pathways. Narrower arrows on either side denote minor pathways.

The antiaggregation pheromone verbenone appears to be produced almost entirely by microorganisms (Hunt and Borden, 1989) (Figure 1), with the beetles' enzymes not involved. This conversion is probably performed by microorganisms in the beetles' galleries, as well as, to a lesser extent, by gut symbionts. Autoxidation of *trans*-verbenol to verbenone supplements the other routes to an unknown but minor extent (Hunt et al., 1989). There is no evidence that verbenone is metabolized further by any system.

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TOXICITY OF ALLELOPATHIC MONOTERPENE SUSPENSIONS ON YEAST
Dependence on Droplet Size

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Abstract—The toxic effects of the allelopathic nonsubstituted monoterpenes β-pinene and limonene on yeast, *Saccharomyces cerevisiae*, were proportional to the size of the monoterpene droplets in suspension. Both the toxic effects and the size of the droplets in suspension were decreased by adding different solvents with the monoterpene as follows: dimethylsulfoxide ≈ dimethylformamide ≫ ethanol > dioxane. Oxygen consumption was inhibited about 80% by 1 mM β-pinene added in dimethylsulfoxide but less than 10% when β-pinene was added in dioxane. Parallel decreases in droplet size and toxic effects of either monoterpene were also induced by hydrating the monoterpene–dimethylformamide or monoterpene–dimethylsulfoxide before addition to yeast. Molecular aggregation may be a mechanism to potentiate the allelopathic properties of monoterpenes when these associate with diverse soil components.

Key Words—Allelopathy, emulsions, monoterpenes, *Saccharomyces cerevisiae*, yeast, suspensions, droplet size, toxicity.

INTRODUCTION

In the soil, monoterpenes are not distributed homogeneously, instead they mix with soil components forming clusters (Muller and Del Moral, 1966). The possibility that the allelopathic properties of the monoterpenes are different depending on the degree to which they are aggregated has not been explored, although reports on the toxic levels of monoterpenes vary widely depending on the experimental conditions.

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Monoterpenes inhibit oxygen consumption by isolated mitochondria from diverse sources (Uribe et al., 1984, 1985; Douce et al., 1978). In a recent report, the effect of β-pinene on several yeast functions was measured (Uribe et al., 1985). Oxygen consumption was inhibited proportionally to β-pinene concentration in intact cells and in isolated yeast mitochondria. For this reason, oxygen consumption by yeast was chosen to test aggregation-dependent toxicity of two cyclic nonsubstituted monoterpenes: β-pinene, a monoene, and limonene, a diene.

The size of the monoterpene droplets in suspension was varied by either using diverse solvents to solubilize each monoterpene (Osipow, 1977) or hydrating the monoterpene–solvent mixture [making a water-in-oil suspension as described in Osipow (1977)] before addition to the yeast incubation medium.

METHODS AND MATERIALS

Reagents. All reagents were of the highest quality available. β-Pinene and limonene were from Aldrich Co.; dimethylsulfoxide (DMSO), dimethylformamide (DMF) and dioxane were from Sigma Co.; ethanol was from Merck. Two-tenths molar β-pinene or limonene was prepared in each solvent (DMF, DMSO, ethanol, or dioxane). Five microliters of these solutions per milliliter of aqueous buffer were added to reach a final 1 mM concentration.

Solvent Hydration. Where indicated, the 0.2 M monoterpene in solvent solutions were hydrated by adding 1–30% (v/v) water (making a water-in-oil suspension) and vortexing. Pertinent volume adjustments were made at the time of addition to the aqueous medium.

Yeast Cell Incubation. Yeast cells from a commercial strain of *Saccharomyces cerevisiae* (La Azteca S.A.) were incubated under aeration for 8 hr in a culture medium and then starved overnight in water. After 24 hr, the cells were washed and resuspended to 50% wet weight per volume in water (Uribe et al., 1985).

Oxygen Consumption. Oxygen consumption was measured with a Clark electrode in a closed chamber equipped with a magnetic stirrer. Temperature was regulated at 30°C. Oxygen concentration in the assay mixture was measured in nanoatoms-gram (natg) (Uribe et al., 1985).

Light Scattering. The light scattering properties of the aqueous monoterpene–solvent suspensions were measured with a fluorimeter using 1-cm light path quartz cuvettes under constant stirring. Light scattering was measured at a 90° angle at 540 nm.

Diphenylhexatriene (DPH) Fluorescence. DPH fluorescence was measured in 20 mM MES-TEA, pH 6.0, buffer with 1 μM DPH and additions of monoterpene in different solvents as indicated. An Aminco spectrofluorimeter

was used with a 340-nm excitation wavelength and 420-nm cutoff filters for emission. Measurements were done at room temperature under constant stirring.

RESULTS

Oxygen consumption by yeast was measured in the presence of 1 mM of either β-pinene or limonene in 5 μl of solvent per milliliter of yeast suspension (Table 1). β-Pinene in DMF or DMSO inhibited oxygen consumption to about 80% of the control rate. When β-pinene was added in ethanol, inhibition of oxygen consumption was only 16% of the control. In the presence of 1 mM β-pinene–dioxane, oxygen consumption was similar to the control rates (Table 1). The effects of 1 mM limonene on the respiratory rate of yeast were tested under the same conditions as β-pinene. In each of the different solvents tested limonene had effects that were similar to those obtained with β-pinene (Table 1).

The effects of 2 mM of either monoterpene in 10 μl/ml of each solvent were also tested (not shown). In DMF, DMSO, and ethanol, inhibition was enhanced. Again, when added in dioxane, very small effects were detected with either monoterpene (not shown).

When added without a solvent, up to 10 mM β-pinene or limonene had no effect on oxygen consumption by yeast (not shown), probably because, when

TABLE 1. EFFECTS OF 1 mM β-PINENE OR LIMONENE IN DIFFERENT SOLVENTS ON OXYGEN CONSUMPTION BY *S. cerevisiae*[a]

Solvent (5 μl/ml)	β-Pinene resp. rate (natgO/min)	Inhibition (%)	Limonene resp. rate (natgO/min)	Inhibition (%)
Control	265 ± 15	0		
Dimethylformamide	51 ± 16	81	52 ± 11	80
Dimethylsulfoxide	32 ± 10	88	29 ± 15	89
Ethanol	206 ± 24	22	195 ± 19	27
Dioxane	238 ± 10	10	243 ± 12	5

[a] Reaction mixture: 20 mM MES-TEA, 10 μl 95% ethanol/ml. A closed water jacketed chamber was used. Temperature, 30°C. Total volume, 3 ml. 1 mM monoterpene was added in 5 μl solvent/ml of reaction mixture as indicated. No additions were made in the control; 25 mg of yeast were added 30 sec after the mixture was prepared. Respiration rate is expressed in nanoatoms-gram of oxygen (natgO) consumed per minute per 25 mg (wet weight) of yeast. Standard deviations ($N = 4$) are included.

added alone, the monoterpenes are immiscible with water and remain on the surface. Addition of each of the solvents by themselves in concentrations of up to five times as much as added with the monoterpenes did not affect oxygen consumption by yeast (not shown).

In order to estimate the size of the droplets in suspension, the light-scattering properties of aqueous suspensions of either monoterpene in each solvent and in the absence of yeast was measured (Osipow, 1977). Figure 1 shows the results obtained with 1 mM β-pinene or limonene in DMF, DMSO, ethanol, or dioxane. The aqueous suspensions of either monoterpene in DMF or DMSO showed much higher light scattering than the monoterpene–ethanol mixture. The monoterpene–dioxane suspensions showed insignificant light scattering. These results indicated a correlation between the size of the aggregates in suspension (Figure 1) and their toxic effects on yeast (Table 1).

An alternate method to estimate the extent of aggregation was to use DPH, a molecule that fluoresces only in hydrophobic environments. The rational for the experiment was that as the monoterpene droplet increased in size so would its hydrophobic core. This method is used commonly for detergent critical micellar concentration detection (Chattopadhyay and London, 1984). Under the conditions employed, DPH did not fluoresce in water or in the solvent–water solutions (not shown). Figure 2 shows the fluorescence of 1 μM DPH in the presence of increasing concentrations of β-pinene in different solvents. DPH

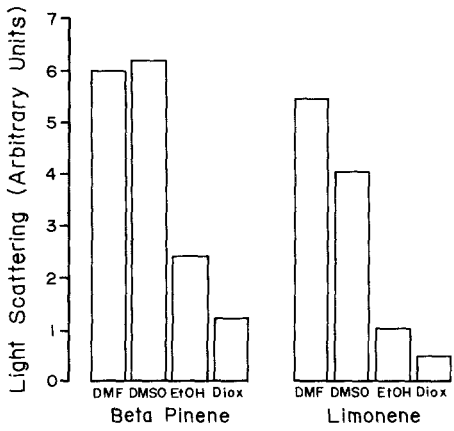

FIG. 1. Light-scattering properties of aqueous suspensions of 1 mM β-pinene or limonene added in DMF, DMSO, ethanol or dioxane. Reaction mixture as in Table 1 except no yeast was added. Room temperature. Monoterpene–solvent additions are indicated in the figure. Readings were taken immediately after mixing.

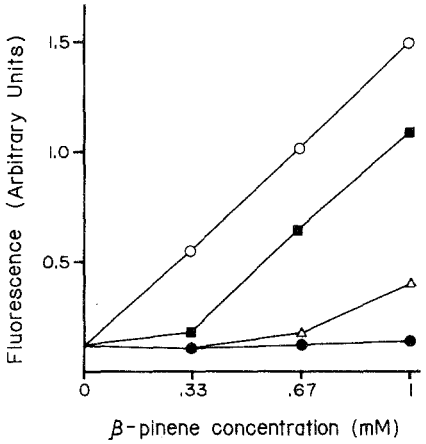

FIG. 2. Diphenylhexatriene fluorescence in the presence of increasing concentrations of β-pinene added in different solvents. Experimental conditions were as in Figure 1, except that the 5-, 10-, or 15-μl β-pinene–solvent mixture was added to the reaction mixture which contained 1 μM DPH. Total volume 3 ml. Solvents were ○, dimethylformamide; ■, dimethylsulfoxide; △, ethanol; and ●, dioxane.

fluorescence increased proportionally to the concentration of β-pinene–DMF or β-pinene–DMSO. There was very little fluorescence with β-pinene–ethanol and even less with β-pinene–dioxane. The same experiment was conducted using limonene instead of β-pinene with similar results (not shown).

Even though the data suggested that monoterpene toxicity was proportional to the size of the droplets in suspension, a direct solvent effect had to be ruled out. To do this, the size of the aggregates in aqueous suspension were modified by hydrating the monoterpene–solvent solutions before addition to the aqueous suspension at a constant final concentration, i.e., adding small amounts of water to a solution of an oil (β-pinene or limonene) in an emulsifying agent (DMF or DMSO) to make a water-in-oil suspension before addition to a large aqueous environment (Osipow, 1977). Figure 3 shows the effect of hydrating a 0.2 M solution of β-pinene–DMF (Figure 3A), β-pinene–DMSO (Figure 3B), limonene–DMF (Figure 3C), or limonene–DMSO (Figure 3D) on the light-scattering properties of the final 1 mM monoterpene–solvent in aqueous suspension.

With β-pinene–DMF, a decrease in light scattering, proportional to the hydration level was observed, decreasing to about one fifth of the control at 30% hydration (Figure 3A). For β-pinene–DMSO, there was a slight increase in light scattering at 4% and 8% hydration, and then at 12% hydration and higher there were larger decreases in light scattering. At 30% hydration, light

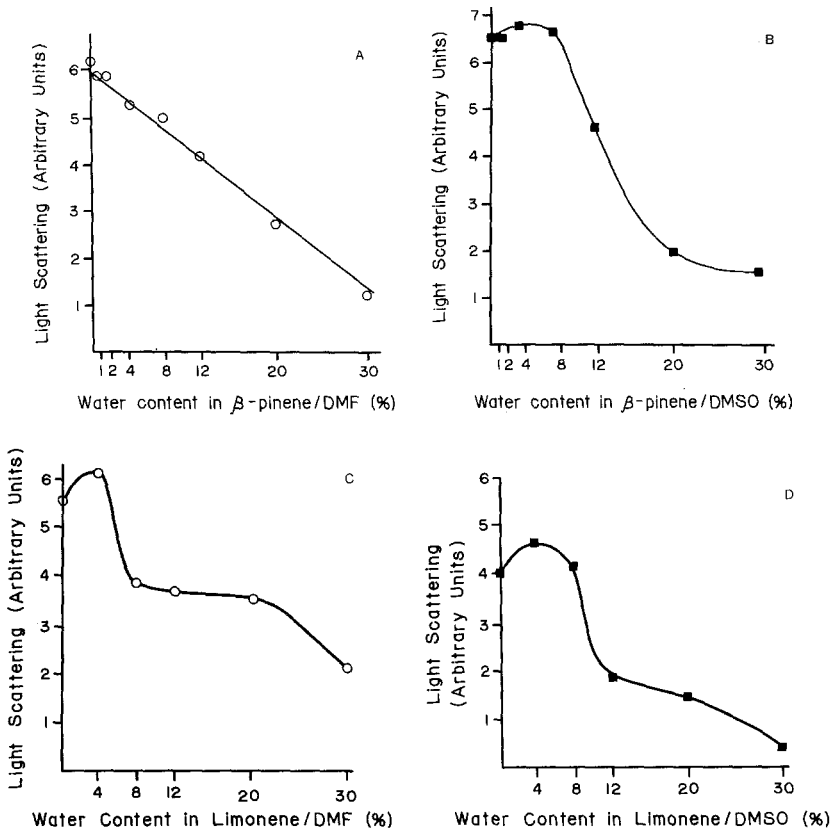

FIG. 3. Light-scattering properties of an aqueous suspension of 1 mM β-pinene or limonene in DMF or DMSO. Effects of previous hydration. Experimental conditions were as in Figure 1. The hydration procedure is described under methods. (A) β-pinene–DMF, (B) β-pinene–DMSO, (C) limonene–DMF, (D) limonene–DMSO.

scattering was about one tenth of the activity of the nonhydrated sample (Figure 3B).

The limonene–DMF system was the least sensitive to hydration because addition of up to 20% water had only discrete effects as compared to the β-pinene mixtures. Even at 30% hydration, the mixture retained almost 40% of the light-scattering activity observed without hydration (Figure 3C). In DMSO, limonene addition resulted in lower light-scattering activity than in DMF. At 30% hydration, light scattering fell to 10% of the nonhydrated mixture (Figure 3D). This value was comparable to the values obtained for β-pinene–DMF or β-pinene–DMSO.

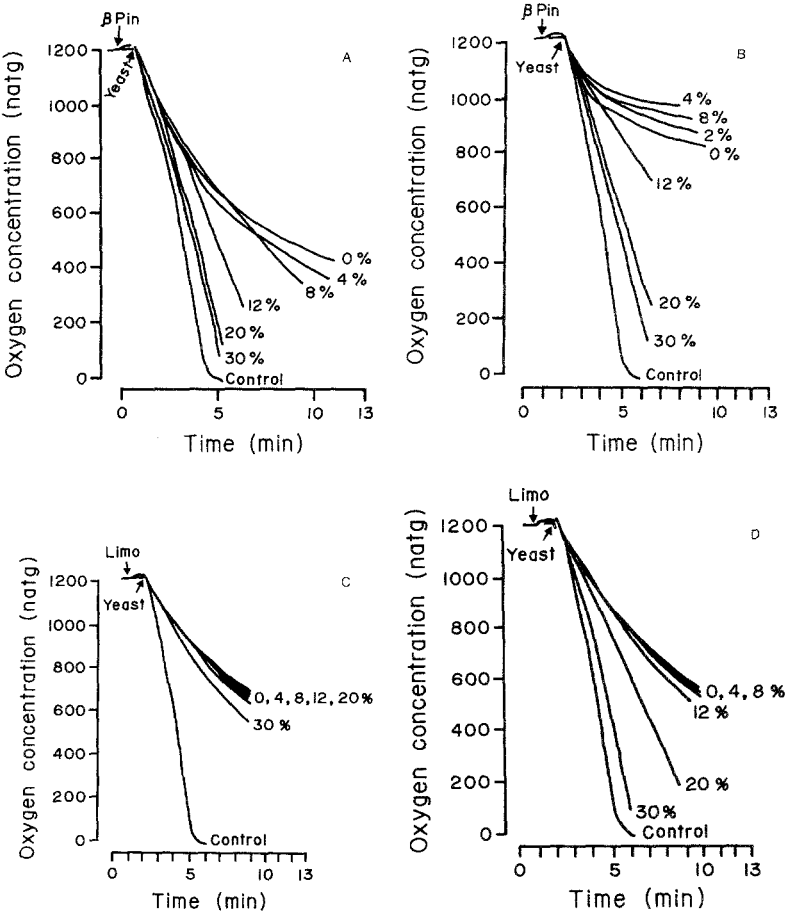

FIG. 4. Effects of 1 mM β-pinene or limonene in DMF or DMSO on yeast oxygen consumption. Dependence on previous hydration. Experimental conditions were as described for Table 1. Numbers are water percentages. (A) β-Pinene–DMF, (B) β-pinene–DMSO, (C) limonene–DMF, (D) limonene–DMSO. The original oxymetry traces are drawn on top of each other. Axes are total oxygen concentration in the medium expressed in nanoatoms-gram (natg) versus time in minutes.

Hydration induced a decrease in the size of the droplets in aqueous suspensions of β-pinene or limonene in either DMF or DMSO. If toxicity were dependent on aggregation, it should also be modified by hydration. Furthermore, these modifications should be smaller for limonene–DMF than for the other monoterpene–solvent mixtures. Figure 4 shows the effects of hydrating the monoterpene–solvent mixtures under the same conditions as in Figure 3 on

the inhibition of oxygen consumption by yeast. In all cases, 1 mM monoterpene in 5 μl of solvent per milliliter of yeast cell aqueous suspension was added.

β-Pinene–DMF-mediated inhibition of oxygen consumption by yeast decreased proportionally with hydration to reach minimal values at 20% and 30% water (Figure 4A). β-Pinene–DMSO inhibition of oxygen consumption increased slightly with hydration up to 8% water and started to decrease at 12%. Inhibition was minimal at 20% and 30% water (Figure 4B). Hydration of 1 mM limonene–DMF to different levels resulted in less reversal of inhibition of oxygen consumption by yeast than that observed by hydration of the β-pinene mixtures (Figure 4C). At 30% hydration, limonene–DMF-mediated inhibition was reduced only slightly. This was expected from the minimal effect that hydration had on the light-scattering properties of the limonene–DMF suspension (Figure 3C). The 1 mM limonene–DMSO inhibition was reversed at 20% and 30% hydration (Figure 4D). Again, this result was expected from the light-scattering data reported above (Figure 3D).

DISCUSSION

Oxygen consumption by yeast was inhibited by β-pinene or limonene. At a fixed monoterpene concentration, this inhibition diminished in parallel to the size of the monoterpene droplets in suspension. Droplet size was modified either by using different solvents or by hydrating the monoterpene–organic solvent solution before addition to yeast. Droplet size variations were estimated by DPH fluorescence (Chattopadhyay and London, 1984) or by measuring the light-scattering properties of the monoterpene–solvent aqueous suspension (Osipow, 1977).

Aggregation dependence of monoterpene toxicity may explain the wide variability on reports of the toxic levels of the nonsubstituted monoterpenes (for a review, see Rice, 1984). Sometimes, only minimal effects at 10 mM or higher are found (Asplund, 1968), while in other systems toxic effects are found at concentrations as low as 30 μM (Asplund, 1968; White et al., 1979). In each report, different vehicles, such as ethanol (Andrews et al., 1980; Muller et al., 1969), corn oil (White et al., 1979), or dimethylformamide (Uribe et al., 1984, 1985), were used. Also, in some cases no emulsifier was used (Asplund, 1968; Koeppel et al., 1981; Douce et al., 1978). Sometimes the solvent used was imposed by the system under study (Clegg et al., 1979), but, in general, the choice was made randomly.

β-Pinene, limonene, and other cyclic monoterpenes are synthesized by higher plants through a light-dependent cyclization of aliphatic monoterpenes (Gambliel and Croteau, 1984; Banthorpe and Njar, 1984). These compounds are expelled to the environment where they exert their allelopathic effects on

microorganisms, insects, and other plants through oxidative phosphorylation inhibition and membrane disruption (Rice, 1984; Andrews et al., 1980; Uribe et al., 1984, 1985).

Aggregation dependence of monoterpene toxicity may be important from a mechanistic point of view because allelopathic monoterpenes are found aggregated with dry soil colloids into droplets where they remain for long periods of time (Muller and Del Moral, 1966). Thus, in their normal environment microorganisms and seeds are exposed to these aggregates and not to homogeneous solutions. Either in the soil or in models such as the suspensions described here, the allelochemical concentration inside each droplet must be extremely high, and therefore toxic effects may be enhanced many times when these droplets come in contact with small areas of biological membranes.

Molecule association resulting in enhancement of some properties has been proposed for many systems such as enzymes traditionally considered as soluble (Srere, 1987) and smaller molecules such as eicosanoids (Fitzpatrick et al., 1984). It is suggested that molecular aggregation may be a mechanism to enhance the allelopathic properties of monoterpenes and perhaps of other compounds. The possibility that the extent of aggregation may modify the biochemical properties of other hydrophobic molecules in aqueous suspension should be kept in mind whenever a hydrophobic substance is used either in vivo or in vitro.

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SEX PHEROMONE COMPONENTS OF FEMALE SMALLER TEA TORTRIX MOTH, *Adoxophyes* sp. (LEPIDOPTERA: TORTRICIDAE) IN TAIWAN

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Abstract—(*Z*)-11-Tetradecenyl acetate (*Z*11–14 : Ac) and (*Z*)-9-tetradecenyl acetate (*Z*9–14 : Ac) were isolated as major sex pheromone components from the female tips of the smaller tea tortrix moth, *Adoxophyes* sp., in Taiwan. The average amount of *Z*11– and *Z*9–14 : Ac in each female gland was 24.9 and 14.1 ng, in a ratio of 64 : 36, respectively. When compared to a closely related Japanese strain, which used the *Z*9–14 : Ac, *Z*11–14 : Ac, *E*11–14 : Ac, 10-Me–12 : Ac four-component system (in a ratio of 63 : 31 : 4 : 2), the Taiwan formulation of two components caught significantly more Taiwan males than the Japanese formulation of four components.

Key Words—Sex pheromone, smaller tea tortrix moth, *Adoxophyes* sp., Lepidoptera, Tortricidae, *Z*9–14 : Ac, *Z*11–14 : Ac, *E*11–14 : Ac, 10-Me–12 : Ac.

INTRODUCTION

In Japan, the female sex pheromone of the smaller tea tortrix moth, *Adoxophyes* sp. (Lepidoptera: Tortricidae), had been identified as a blend of (*Z*)-9-tetradecenyl acetate (*Z*9–14 : Ac), (*Z*)-11-tetradecenyl acetate (*Z*11–14 : Ac), (*E*)-11-tetradecenyl acetate (*E*11–14 : Ac), and 10-methyldodecyl acetate (10-Me–12 : Ac) in a ratio of 63 : 31 : 4 : 2 (Tamaki et al., 1979, 1980), but few males were caught with this ratio in a tea plantation on Taiwan (Shry and Chow, 1985). Pheromone polymorphism (different strains) might occur, so the chem-

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ical constituents of the sex pheromone of the female smaller tea tortrix moth in Taiwan were investigated.

METHODS AND MATERIALS

Insects. Pupae of the smaller tea tortrix moth used in this study were supplied by the Taiwan Tea Experiment Station, Hsinchu, Taiwan. Male and female pupae were held in separate rooms at 23–26°C under continuous lighting.

Preparation of Ovipositor Extracts. Ovipositors from 2000 3- to 4-day-old virgin females were excised and immersed in hexane for 30 min. The extracts were stored at 0°C for bioassay and structure identification.

Bioassay of Crude Extract. Five 3-day-old males, which had been conditioned under continuous light after emergence, were put into a 250-ml flask and preconditioned in the dark phase for 7 hr before bioassay. The bioassay method was based on Tamaki et al. (1969). The experiment was repeated four times.

Fractionation of Ovipositor Extract. The fractionation procedure of the crude ovipositor extract was the same as described by Tamaki et al. (1969).

GC and GC-MS Analysis. Gas chromatography (GC) was performed with a Varian 3700 GC equipped with a flame ionization detector. Other chromatographic conditions are described in Table 1. Gas chromatographic–mass spectrometric (GC-MS) analysis was conducted as described previously (Kou et al., 1989).

The sex pheromone components were identified by comparison of their mass spectra with those of authentic standards. Subsequently, the identification was verified by comparison of the GC retention of the natural product to that of an authentic standard using the four capillary columns under the same GC conditions as described in Table 1. Standards of *Z*9–14 : Ac, *Z*11–14 : Ac, *E*11–14 : Ac, and 10-Me–12 : Ac were purchased from Sigma Chemical Co.

Pheromone Titer Determination. One thousand 3- to 4-day-old virgin

TABLE 1. GAS CHROMATOGRAPHIC CONDITIONS

Column	Temperature	Carrier gas
30 m × 0.25 mm DB-1, J & W	80–240°C 4°C/min	8 psi, He
35 m × 0.53 mm Carbowax 20 M, Analabs	100–200°C 4°C/min	7 psi, He
30 m × 0.25 mm DB-17, J & W	130–250°C 4°C/min	10 psi, He
30 m × 0.32 mm SP-2340, Supelco	100–240°C 4°C/min	8 psi, He

females' ovipositors were excised and pooled together in hexane during their calling period. In this prepared, pooled extract, each microliter of solution contained 2.5 ovipositors and 0.05 μg (*Z*)-11-hexadecenyl acetate (*Z*11–16 : Ac) as an internal standard. The extract was subsequently analyzed for *Z*9–14 : Ac and *Z*11–14 : Ac using the internal standard method of quantitative analysis.

Field Trapping of Released Males. Field trapping with Taiwan virgin females and synthetic chemicals with different blends of *Z*11–14 : Ac and *Z*9–14 : Ac (64 : 36, Taiwan formulation) and *Z*9–14 : Ac, *Z*11–14 : Ac, *E*11–14 : Ac, and 10-Me–12 : Ac (63 : 31 : 4 : 2, Japanese formulation) was carried out at a test field near the Academia Sinica, Taipei, during May 4–9 and May 22–27, 1989. One and 0.2 mg synthetic chemicals were dissolved in hexane and loaded in plastic capsules. The plastic capsule was hung on the inside at the top of a wing-shaped sticky trap. Blank traps and traps baited with four virgin females per trap were used for comparison. Traps were placed 10 m apart and 1.5 m above ground and randomized nightly. A total of 1075 laboratory reared 3- to 4-day-old unmated males were released. Each trap was checked each day for six consecutive days.

Field Testing. Field testing of the two-component Taiwan formulation and the four-component Japanese formulation was carried out in a tea plantation in Nankang, Taipei, during August 21–September 3, 1989, according the above method.

Duncan's new multiple-range test (Steel and Torrie, 1960) was used to analyze the field test results.

RESULTS AND DISCUSSION

Laboratory Bioassay. The results of the bioassay with Taiwan female crude extract are shown in Figure 1. The peak of male response was at 10^{-3} FE and then decreased with further increased concentrations.

Fractionation and GC-MS Analysis. Activity in laboratory bioassays was obtained with fractions eluted with 2% and 4% ether from the Sep-Pak silica cartridge. GC analysis showed that each of the active fractions (Figure 2) contained two major peaks. Previously reported sex pheromone components for the smaller tea tortrix moth include *Z*9–14 : Ac, *Z*11–14 : Ac, *E*11–14 : Ac, and 10-Me–12 : Ac (Tamaki et al., 1980). These four compounds were prepared for comparison, and it was found that synthetic *Z*9–14 : Ac and *Z*11–14 : Ac had the same retention times (Table 2) and mass spectra in all four columns used as the two major peaks isolated from ovipositor extracts.

Pheromone Titer Determination. The amount of the compounds identified as *Z*11–14 : Ac and *Z*9–14 : Ac was determined to be 39.0 ng/ovipositor in a ratio of 64 : 36, respectively, from ovipositors of 3- to 4-day-old Taiwan virgin

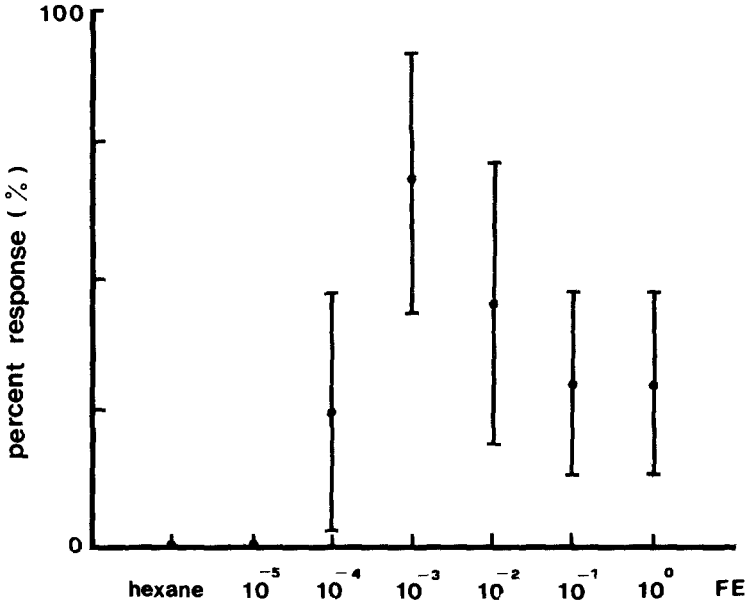

FIG. 1. Sexual response of male *Adoxophyes* sp. to different female equivalent (FE) crude extract. The vertical bars represent ± standard error of each mean.

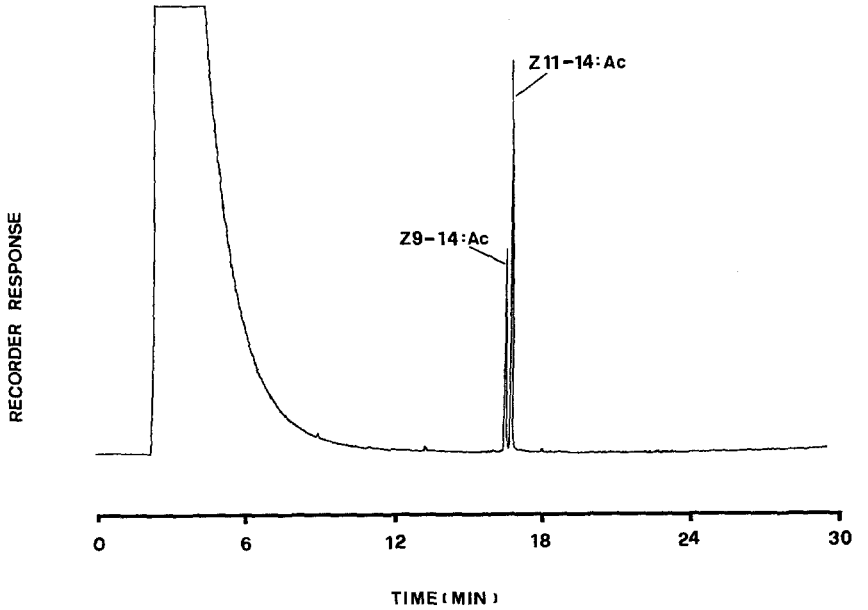

FIG. 2. Gas chromatograms of the female crude extract and the fraction eluted with 4% ether in hexane from Sep-Pak cartridge (Waters Associates). The gas chromatography was performed on a 30-m × 0.25-mm DB-17 column temperature programmed from 130 to 250°C at 4°C/min.

TABLE 2. RETENTION TIMES OF 10-Me-12:Ac, Z9-14:Ac, Z11-14:Ac, E11-14:Ac, AND SEX PHEROMONE EXTRACT OF FEMALE *Adoxophyes* sp. ON FOUR GAS CHROMATOGRAPHIC COLUMNS

Column type	Retention time (min)				
	1-Me-12:Ac	Z9-14:Ac	Z11-14:Ac	E11-14:Ac	Extract
DB-1	28.00	31.25	31.52	31.61	27.97 31.21 31.53
Carbowax 20 M	8.62	12.11	12.49	12.30	8.61 12.10 12.48
DB-17	13.14	16.30	16.49	16.40	16.27 16.48
SP-2340	9.37	12.63	12.81	11.56	12.65 12.90

females, as shown in Table 3. This ratio was not changed after passing the crude extract through a Sep-Pak cartridge (Figure 2).

Field Trapping of Released Males. The results of field trapping of released males are shown in Table 4. The attractiveness of 1 mg synthetic pheromone with a 64:36 blend ratio of Z11-14:Ac and Z9-14:Ac (Taiwan formulation) is significantly higher than the Japanese formulation and virgin females ($P < 0.05$).

Field Trapping of Wild Males. The results of field trapping of wild males from a tea plantation are shown in Table 5. Again, the attractiveness of 1 mg synthetic pheromone with the Taiwan formulation is significantly higher than with the Japanese formulation ($P < 0.05$).

In this study, the sex pheromone blend ratio of the smaller tea tortrix moth in Taiwan (Z11-14:Ac and Z9-14:Ac, 64:36) was found to be reversed from that of the Japan smaller tea tortrix moth (Z9-14:Ac and Z11-14:Ac, 63:31). In the field, the response of Taiwan strain males to the Taiwan formulation was

TABLE 3. AVERAGE QUANTITY (ng/♀) AND PERCENTAGE OF Z9-14:Ac AND Z11-14:Ac PRODUCED BY 3- TO 4-DAY-OLD *Adoxophyes* sp. VIRGIN FEMALES

Component	Quantity (ng)	Percentage (%)
Z11-14:Ac	24.9	63.8
Z9-14:Ac	14.1	36.2
Total	39.0	100

TABLE 4. NUMBER (MALES/TRAP/DAY) OF RELEASED *Adoxophyes* sp. MALES ATTRACTED TO DIFFERENT SEX PHEROMONE BLEND RATIOS OF TAIWAN FORMULATION (Z11-14 : Ac/Z9-14 : Ac = 64 : 36) AND JAPANESE FORMULATION (Z9-14 : Ac/Z11-14 : Ac/*E*11-14 : Ac/10-Me-12 : Ac = 63 : 31 : 4 : 2), AND VIRGIN FEMALES, MAY 4–9 AND MAY 22–27, 1989

Date	Z11-14 : Ac/Z9-14 : Ac (64 : 36)		Z9-14 : Ac/Z11-14 : Ac/ *E*11-14 : Ac/10-Me-12 : Ac (63 : 31 : 4 : 2)			
	1 mg	0.2 mg	1 mg	0.2 mg	Virgin ♀	Control
May 4	24	16	8	1	6	0
5	25	10	8	7	21	0
6	3	4	4	2	15	0
7	15	6	1	2	2	0
8	4	2	0	0	3	0
9	0	4	1	1	3	0
22	15	1	1	1	8	0
23	26	7	4	1	10	0
24	16	6	13	5	6	0
25	9	4	2	0	5	0
26	30	5	29	8	17	0
27	25	5	14	0	14	0
$\bar{X}$ (males/trap/day)[a]	16.0 ± 9.8 a	5.8 ± 3.8 bc	7.1 ± 8.0 bc	2.3 ± 2.7 cd	9.2 ± 5.9 b	0 d

[a] Means followed by different letters are significantly different. ($P < 0.05$, Duncan's new multiple-range test).

TABLE 5. NUMBER (MALES/TRAP/DAY) OF *Adoxophyes* sp. MALES ATTRACTED TO DIFFERENT SEX PHEROMONE BLEND RATIOS OF TAIWAN FORMULATION (Z11-14 : Ac/Z9-14 : Ac = 64 : 36) AND JAPANESE FORMULATION (Z9-14 : Ac/Z11-14 : Ac/ *E*11-14 : Ac/10-Me-12 : Ac = 63 : 31 : 4 : 2) AUGUST 21–SEPTEMBER 3, 1989

Date	Z11-14 : Ac/Z9-14 : Ac (64 : 36)		Z9-14 : Ac/Z11-14 : Ac/*E*11-14 : Ac/10-Me-12 : Ac (63 : 31 : 4 : 2)	
	1 mg	0.2 mg	1 mg	0.2 mg
Aug. 21	4	0	1	0
22	5	2	1	0
23	8	0	0	1
24	6	1	1	0
25	3	1	1	1
26	16	3	4	1
27–28	10	2	1	0
29–30	15	6	4	0
Aug. 31–Sep. 1	6	2	1	1
Sep. 2–3	12	2	3	1
$\bar{X}$ (males/trap/day)	8.5 ± 4.3 a	1.9 ± 1.6 b	1.7 ± 1.3 b	0.5 ± 0.5 b

[a] Means followed by different letters are significantly different. ($P < 0.05$, Duncan's new multiple-range test).

significantly higher than to the Japanese formulation. Thus the occurrence of pheromone polymorphism (different strains) was assumed. The classical case of pheromone polymorphism is represented in the European corn borer, *Ostrinia nubilalis*. Kochansky et al. (1975) verified the existence of two strains in North America, one in New York (E strain) with a ratio of 4 : 96 of *Z*11–14 : Ac and *E*11–14 : Ac, and one in Ontario (Z strain) with a 97 : 3 ratio, which resembled the population in Iowa (Klun and Robinson, 1971).

We should determine if minor components exist in the sex pheromone system of the smaller tea tortrix moth in Taiwan.

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HYDRODYNAMIC CONSTRAINTS ON EVOLUTION OF CHEMICALLY MEDIATED INTERACTIONS BETWEEN AQUATIC ORGANISMS IN UNIDIRECTIONAL FLOWS

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Abstract—Two hydrodynamic habitats can be defined in unidirectional flow: (1) A boundary where molecular diffusion dominates is found within roughly 1 mm of solid substrates. The actual thickness of this diffusion boundary is a function of current velocity and topography of the substrates. (2) A zone of turbulent flow is present outside the diffusion boundary where chemicals are rapidly diluted and transported downstream. The mode of chemically mediated interaction between organisms in streams is constrained differently depending on which of these two habitats they occupy. Within a diffusion boundary, reciprocal interaction between small organisms is possible because mixing is low, diffusion flux high, and organisms "share" the same water. Outside a diffusion boundary, in unidirectional flow, organisms unable to move effectively against flow are only chemically influenced by upstream organisms and can only influence downstream organisms. Organisms that are able to move upstream can interact reciprocally with other organisms, even if one or both organisms are found primarily in areas of turbulent mixing.

Key Words—Allelochemicals, chemically mediated interaction, competition, nutrients, streams, diffusion boundary, directional flow.

INTRODUCTION

Hydrodynamic properties constrain the types of chemically mediated interactions between organisms in streams because hydrodynamics control diffusional flux of dissolved chemicals. With unidirectional flow over benthos, water movement varies from high current velocities away from the substrate to extremely low velocities in the areas near the substrate (the diffusion boundary).

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In this paper, I discuss the functional differences between chemically mediated organismal interactions within and outside of the diffusion boundary.

Literature review revealed that few data on chemically mediated interactions between stream organisms are available. However, there are many well-documented cases of chemically mediated interactions between terrestrial organisms and between marine organisms. On the basis of hydrodynamic constraints, I hypothesize that such interactions between organisms in streams can be reciprocal inside the diffusion boundary and are only unidirectional outside the boundary unless the organisms involved can move upstream against current. Interaction is viewed from the perspective of the individual organism because it can be reciprocal at the population or species level and still be unidirectional at the individual level. Where it is possible, the hypothesis is supported with direct evidence; where it is not possible, evidence from marine or terrestrial environments is considered.

The discussion centers on streams but is also applicable to estuarine systems and other aquatic benthos experiencing regular, unidirectional currents. I attempt to discuss all types of chemically mediated interactions between organisms that may occur in streams. How hydrodynamics create subhabitats will be considered first, organismal interactions mediated by inorganic chemicals second, and interactions controlled by organic chemicals third. The relationship between the relative concentrations of chemicals and the type of interactions will then be discussed, followed by conclusions and recommendations for future research.

HYDRODYNAMICS AND STREAM SUBHABITATS

To follow the arguments in this paper, it is necessary to understand some basic properties of flow. Streams are divided into two subhabitats: those in the diffusion boundary and those outside the diffusion boundary. A diffusion boundary in which molecular diffusion dominates usually surrounds solid substrates in unidirectional flow. This boundary is dominated by molecular diffusion because it is rarely interrupted by turbulent flow. However, the diffusion boundary is not always present, particularly when velocity is high and flow very turbulent. The thickness of the diffusion boundary can vary; it decreases as current velocity increases and as the substrate protrudes into flow. Generally the diffusion boundary is < 1 mm thick (Nowell and Jumars, 1984).

Areas away from the diffusion boundary are dominated by turbulent flow and are characterized by mixing or eddy diffusion. With eddy diffusion, total chemical flux is lower than in the diffusion boundary because of dilution into larger volumes of water, but the rate of chemical transport is higher because mixing moves individual molecules much more rapidly than molecular diffu-

sion. To simplify discussion, flow outside the diffusion boundary is assumed to be unidirectional, although turbulence and eddies can cause small regions of upstream flow in streams.

The diffusion boundary should not be confused with the flow boundary (Silvester and Sleigh, 1985). Flow boundaries (viscous sublayers) are the regions in which viscous forces constrain movement of water molecules. Within the flow boundary, current decreases relative to open-channel current velocity. In this boundary, friction with the substrate causes flow to slow and become more laminar (Silvester and Sleigh, 1985). The diffusion boundary is smaller than the flow boundary because some turbulent mixing occurs on the outer edge of the flow boundary, causing eddy diffusion to exceed molecular diffusion. Hydrodynamic factors influence flow boundary and diffusion boundary thickness in the same manner (i.e., thickness decreases with increased current velocity and as the substrate protrudes into the flow), even though the diffusion boundary is thinner.

Silvester and Sleigh (1985) describe how the thickness of the flow boundary changes with varied flow around selected structures, and Statzner and Holm (1989) have made actual microcurrent measurements around stream organisms that verify the theoretical discussion of Silvester and Sleigh (1985) and illustrate the complexity of fluid dynamics in natural environments. For a more complete discussion of the mathematics of flow, consult the following references: Butman (1986), Grant and Madsen (1986), Nowell (1983), and Nowell and Jumars (1984). The mathematics of diffusion, especially with regard to turbulent flows, are covered in general terms in: Berner (1980), Okubo (1980), Vogel (1981), and with respect to unidirectional flow in Fischer (1973).

INTERACTIONS MEDIATED BY INORGANIC CHEMICALS

Interactions between organisms controlled by inorganic chemicals are divided into two types. Negative interactions are generally dominated by nutrient competition. Positive interactions are related to nutrient regeneration and utilization of harmful inorganic compounds.

Negative Interactions. Nutrient competition is the most important negative inorganic chemically mediated interaction between organisms in aquatic ecosystems and is governed by relative uptake rates. The relevance of a plant's nutrient uptake capacity to its competitive ability was described by Tilman (1982). Data for uptake capacity of autotrophs found in streams are scarce, but the interrelationships described by Tilman (1982) can be applied to nutrient competition by stream organisms as long as the constraints of flow and diffusion are considered.

Within a diffusion boundary intra- and interspecific nutrient competition

are reciprocal (see Table 1 for summary of general interactions). Periphyton and associated heterotrophs in streams can exist within a diffusion boundary where molecular diffusion dominates (Silvester and Sleigh, 1985) and should generally exhibit reciprocal interaction. Specifically, competition between epiphytes and their macrophytes is reciprocal (Fitzgerald, 1969), and epiphytes can be inside the diffusion boundary surrounding macrophyte leaves (Jørgensen and Revsbech, 1985).

Spatial and temporal heterogeneity of nutrient concentration within a diffusion boundary may influence the specific manifestation of reciprocal nutrient competition. Cells create a halo of decreased nutrient concentration around themselves (Whitford and Schumacher, 1961), and heterotrophs create microzones of regenerated nutrients (Lehman and Scavia, 1982) that may persist within a diffusion boundary, but would rapidly disperse with turbulence (Jackson, 1980).

Motile organisms within a diffusion boundary can move to areas of higher nutrient concentration, giving them a distinct advantage over attached organisms when nutrients are spatially heterogeneous. Gliding motility allows microorganisms to move into optimal subhabitats, which could explain the prevalence of pennate diatoms on solid substrates in streams. Unstalked pennate diatoms are able to glide and rarely protrude outside of the diffusion boundary.

The reciprocal nature of chemically mediated interactions within the diffusion boundary, therefore, has resulted in several different strategies for nutrient competition. Selection on cells that grow in a diffusion boundary probably favors those able to grow at relatively high rates at ambient nutrient concentrations (have a low $\frac{1}{2}$ saturation constant at low nutrients, or a high maximum uptake

TABLE 1. SUMMARY OF FACTORS INVOLVED IN CHEMICALLY MEDIATED INTERACTIONS WITHIN AND OUTSIDE A DIFFUSION BOUNDARY

Factor	Inside	Outside
Diffusion	molecular	eddy
Dilution	low	high
Diffusion flux	high	low
Spatial scale (distance between interacting species)	small (< ca. 1 mm)	large (> 1 mm)
Organism size	small	large
Direction of interaction	all directions	unidirectional
Concentration of excreted chemical reaching interacting species	high	low
Allelochemical inhibitors	more effective	less effective
Attractants	chemotaxis	emitted in plumes
Nutrient competition	reciprocal	indirect

at high nutrients; Tilman, 1982), those that can take advantage of spatial heterogeneity in nutrient supply via luxury consumption, those able to use nutrient forms unavailable to others (i.e., N_2 fixation and other types of specialized metabolism), or motile organisms able to move to nutrient-rich areas (Kelly et al., 1988).

In contrast to organisms within the diffusion boundary, reciprocal nutrient competition between organisms separated by unidirectional turbulent flow does not exist. Rather, upstream primary producers incorporate nutrients, which then become unavailable to downstream producers until the nutrient is remineralized. This is nutrient spiraling (Elwood et al., 1983), where nutrients that are remineralized spiral downstream and are not returned to the same primary producers that initially incorporated the nutrients.

Positive Interactions. Organisms can release nutrients that are essential to other organisms, or they can decrease concentrations of inorganic chemicals that have negative effects on other organisms. As with nutrient competition, positive interactions involving inorganic chemicals are expected to be reciprocal within the diffusion boundary and unidirectional in areas of flow.

An example of positive reciprocal interaction within a diffusion boundary is the association between bacteria and heterocystous cyanobacteria (blue-green algae). The bacteria benefit from dissolved organic carbon or nitrogen compounds that leak from the N_2-fixing heterocysts. The cyanobacteria presumably are able to fix N_2 more efficiently as a result of bacterial respiration, which lowers O_2 tension around them (Paerl, 1985). In another case of positive interaction, the midge larva *Cricotopus nostocicola* eats and lives inside the cyanobacterium *Nostoc parmelioides*. Larval respiration supplies CO_2 and lowers O_2 concentration, which increases the photosynthetic rate of the cyanobacterium (Dodds, 1989).

Positive unidirectional interactions between organisms in areas outside the diffusion boundary involve organisms that increase nutrient availability to primary producers downstream. Remineralization by macrophytes (Twilly et al., 1985) and bioturbation by aquatic insects (Merritt et al., 1984) can increase the rate at which nutrients enter streams from sediments. Beavers can increase the rate nutrients enter streams from riparian zones (Naiman et al., 1986; Dodds and Castenholz, 1988).

INTERACTION MEDIATED BY ORGANIC CHEMICALS

Hydrodynamics similarly constrain organismal interactions mediated by organic and inorganic chemicals. Two types of interactions controlled by organic chemicals are considered: allelochemical and behavioral. I use the definition of Lewis (1986), which states that allelochemicals are organic metabolites of one

organism that suppress or stimulate growth of another organism. Under this definition, all other interactions involving organic chemicals are behavioral. This is an arbitrary dichotomy because some chemicals are involved in both allelochemical and behaviorally mediated interactions. For example, toxic chemicals may be used by grazers as behavioral cues (i.e., they may taste bad). Although other investigators may view behavioral cues as allelochemicals (Lovett et al., 1989), acceptance of Lewis' (1986) definition will clarify the following arguments.

Types of Allelochemicals. Freshwater organisms produce allelochemicals. They are excreted by planktonic bacteria and phytoplankton (Gantar, 1985; Keating, 1977) and by benthic macrophytes and unicellular algae (Sharma, 1985; Wium-Andersen et al., 1982). Allelochemicals also can remain sequestered inside individual cells as a defense against grazing or predation (Gregory, 1983; Lamberti and Moore, 1984; Lubchenco and Gaines, 1981).

There are two fundamentally different types of allelochemical interaction: (1) excretory by-products that are used as environmental cues and trigger either increased or decreased growth in the receptor organism, and (2) excretory chemicals that are not solely by-products but are synthesized for the express purpose of raising or lowering the growth of other organisms. Both types of allelochemical interaction will be discussed with respect to organisms within and outside the diffusion boundary.

Allelochemical Interaction outside the Diffusion Boundary. Lewis (1986) argued that allelochemicals produced by plankton should be excretory by-products that are used by other organisms as growth signals or cues to environmental conditions. The argument is also applicable to allelochemical interaction between organisms separated by unidirectional flow. It is possible for organic excretory products to be used as growth cues by downstream organisms. The only selection that occurs is on the organisms that use these products as cues. It is unlikely that large sessile organisms (i.e., macrophytes) would synthesize and release allelochemicals to inhibit growth of downstream organisms. It is energetically expensive to produce such chemicals, and the only organisms affected would be downstream. As discussed above, downstream organisms are not competitors for resources (nutrients, light, or space). Therefore, there is no selection for sessile organisms to produce energetically expensive allelochemicals because there is no benefit to interfering with growth of downstream organisms.

Allelochemical Interaction within a Diffusion Boundary. Small organisms within a diffusion boundary may produce specific chemicals to inhibit the growth of competitors (the second type of allelochemical discussed above). Organisms within the boundary are competing directly with the surrounding cells for resources (Murray et al., 1986) and can directly affect the cells around them via molecular diffusion. There are no data on such interactions within periphy-

ton communities in aquatic systems, but inhibitory interactions do occur between macrophytes and their epiphytes. Epiphytes are within the diffusion boundary surrounding macrophyte leaves and thus experience high concentrations of any exudates. They are also in direct competition with their host macrophytes for light and nutrients, and the cost to macrophytes of producing chemicals that inhibit epiphyte growth is immediately repaid; thus there is a selective benefit to synthesizing allelochemicals for the express purpose of lowering epiphyte growth. This interaction in estuarine systems was documented by Harrison and Durance (1985), who showed that water-soluble extracts from leaves of *Zostera marina* L. inhibited photosynthesis of epiphytic diatoms. Harrison (1982) showed that *Zostera marina* extracts inhibited microbial growth. Wium-Anderson et al. (1982) documented that freshwater macrophytes (Charales) excrete dithiolane, trithiane, and elemental sulfur, all compounds that reduce epiphyte growth.

Organic chemicals that positively affect growth of other organisms can be excreted within a diffusion boundary. For example, bacterial growth within biofilms can be stimulated by organics excreted by photosynthetic organisms (Murray et al., 1986).

Allelochemicals can be used by organisms as protectants from grazing or predation. One strategy is to sequester chemicals that make an organism toxic to its predator, either inside or at the outer surface. Such compounds are probably sequestered by aquatic macrophytes (Otto and Svensson, 1981); aquatic Hemiptera, Coleoptera, and other insects (Scrimshaw and Kerfoot, 1987); and some rotifers, protozoa, and cladocera (Havel, 1987). The effectiveness of these compounds is dependent upon maintaining high concentrations. High diffusion rates may increase the cost of maintaining effective concentrations. Sequestered compounds in organisms exposed to extremely turbulent stream flow should be less water-soluble than in lentic organisms, which experience less severe turbulence. Hepburn et al. (1973) have shown that water-insoluble compounds are the most effective feeding deterrents for fish.

Macrophytes in streams may be more likely to sequester allelochemical compounds than those in lentic waters, according to the hypothesis presented by Ott and Maurer (1977) on the indirect selective pressure of herbivores. When herbivores regenerate nutrients that are not transported away from grazed plants (i.e., in small lakes), there may be an advantage to macrophytes that allow herbivores to liberate nutrients from less productive, older plant tissue. Regenerated nutrients in streams are transported downstream. Therefore, there may be stronger selective pressure to prevent grazing on macrophytes in flowing water than in lentic systems.

Behavioral Cues as Protection from Predation and Grazing within the Diffusion Boundary. Some chemical behavioral cues "advertise" unpalatability. Grazing and predatory fish rely upon food taste to determine feeding preferences

(Adams and Johnson, 1985), as do copepods (Butler et al., 1989) and aquatic insects. Both unpalatable and sequestered compounds are used as protection simultaneously, except in cases of chemical mimicry. There is an advantage to advertising toxicity to potential grazers or predators because cells must be damaged before sequestered compounds are effective. These compounds are excreted at low levels or tightly bound to the surface of the "advertising" organism so that they are only detectable within the diffusion boundary. Using this strategy, the "advertiser" is not injured by nearby grazers or predators and can avoid spreading a large plume of attractant that may be detected by other organisms able to overcome its defenses. Moreover, excretion of excess amounts of the "advertising" chemical would be energetically wasteful and should be selected against.

Sticky or entangling defensive exudates are common in terrestrial insects (Blum, 1981). Such exudates may be produced by stream organisms if water does not render them ineffective. Water-soluble defensive exudates would probably not work because they would be rapidly diluted (Hepburn et al., 1973).

Soluble Repellents and Attractants within the Diffusion Boundary. Organic chemicals released by single cells as attractants within diffusion boundaries may attract motile cells by conventional models of chemotaxis (Levandowsky and Hauser, 1978; Cooksey and Cooksey, 1986; Levandowsky et al., 1988). Chemotactic relationships between microorganisms include predator–prey interaction, mating, mutualisms, and aggregation phenomenon (Chet and Mitchell, 1976). The diffusion boundary is actually extended by *Oedogonium*, which excretes a gel from oogonia that facilitates chemical attraction of sperm (Rawitscher-Kunkel and Machlis, 1962).

Except in low flow, it is unlikely that small organisms could swim faster than the current velocity. If an organism is able to swim faster than the current velocity, it may be able to utilize chemical attractants released into areas of flow. It is doubtful that cells that form biofilms are attracted to a specific site before they enter a diffusion boundary. Because of the relatively low swimming speed at their dispersal stages (Breznak et al., 1984), small organisms that mainly reside within the diffusion boundary passively colonize attachment sites by hydrodynamic processes.

Soluble Repellents and Attractants outside the Diffusion Boundary. Organic chemicals can be used as attractants in streams. Chemical cues are common in all animals and are the most primitive form of communication (Kitteridge et al., 1974). Chemical attractants may be most strongly selected for in streams where visibility is limited by high turbidity, in deep parts of rivers that receive little light, at night, or over long distances where vision is unimportant.

The high dilution of turbulent flow rapidly spreads attractants. In areas of unidirectional flow, diffusion causes a plume of attractant (Okubo, 1980). Models have proposed an upwind zigzag search pattern for flying insects fol-

lowing a plume of attractant created by unidirectional air flow (Cardé, 1984). Similar models are probably applicable to many swimming organisms and have been documented for fish (Johnson, 1986). These models require the organism to have a "memory" of the chemical cue and the ability to sense current and movement relative to the substrate. Behavior of terrestrial walking insects following an airborne plume of attractant has also been described (Bell, 1984). Similar behaviors are probably exhibited by stream organisms that remain in constant contact with the stream bottom but are large enough to protrude out of a diffusion boundary.

Many types of aquatic organisms follow chemical attractants. Snails that graze epiphytes from *Ceratophyllum demersum* were able to follow chemical attractants and find undamaged leaves in still water (Bronmark, 1985). Lobster behavior is chemically mediated (Zimmer-Faust and Case, 1983), and the planktonic shrimp *Acetes sibogae australis* is attracted by low concentrations of some amino acids (Hamner and Hamner, 1977). Protistans exhibit chemosensory behavior (Verity, 1988; Levandowsky et al., 1988). Chemical communication has been shown to be important in the reproduction, homing, feeding, schooling, and parent–young interactions of fish (Johnson, 1986), and salmon use olfaction to return to spawning grounds (Schulz et al., 1976).

Conversely, organic chemicals may act as repellents. Some mayflies avoid regions downstream from their stonefly predators (Peckarsky, 1980), probably a chemically mediated response. Aquatic insects may also avoid their stonefly predators by chemotactile mechanisms (Peckarsky and Dodson, 1980), and amphipod predation–avoidance behaviors in response to chemical cues released by fish have been documented (Williams and Moore, 1985). Furthermore, *Daphnia* (Grant and Bayley, 1981; Dodson, 1989), mosquito larvae (Sih, 1986), minnows (Lawrence and Smith, 1989), and larval amphibians (Petranka et al., 1987) respond to chemical cues, indicating the presence of predators.

INTERRELATIONSHIPS BETWEEN CHEMICALLY MEDIATED INTERACTION AND CONCENTRATIONS

Physiological or behavioral cues result from qualitatively different selective processes than do growth substrates and compounds that inhibit growth of competitors or predators. The different selective processes can be used to predict the relative concentration at which chemically mediated interaction occurs (Lovett et al., 1989). This concentration is a function of both rates of production and hydrodynamically influenced diffusion.

Chemicals used as behavioral or physiological cues are likely to be important in areas of turbulent flow as well as within the diffusion boundary because they can be effective at low and high concentrations. Selective pressure is for

the lowest possible receptor threshold for behavioral and physiological cues. Therefore, there is selection for organisms able to sense the highly diluted chemical cues associated with turbulent flows. The threshold detection limit for chemicals that signal unpalatability to fish exuded by arthropods is around $1.0–6.0 \times 10^{-6}$ M (Hepburn et al., 1973): coho salmon can be attracted to a breeding site with concentrations of morpholine from 1.1×10^{-6}–1.1×10^{-7} M (Schulz et al., 1976), cnidarian nematocyst discharge responses are chemosensitized by $< 10^{-6}$ M acetylated sugars and mucin (Watson and Hessinger, 1989), and planktonic marine shrimp are attracted to $< 10^{-4}$ M L-methionine (Hamner and Hamner, 1977). The ability to sense low concentrations of the cues makes it possible for such interactions to occur in spite of the high dilution found outside of the diffusion boundary.

Toxic chemicals are effective at widely variable concentrations. Greater than 1000-fold variations in the effects of allelochemicals on arthropods (Mullin, 1986) have been observed within species. As Janzen (1979) wrote, "one beast's drink is another beast's poison." The variability in effective concentration is a result of coevolutionary processes. Selection favors the organism that releases the most toxic compound because production of a smaller amount of a more toxic compound is the most energy-efficient strategy. Conversely, resistance to the toxin is selected for in the target organism, so only high concentrations are effective. Therefore, some toxic compounds may be effective at the high dilution of turbulent flow, but the higher concentrations found within a diffusion boundary may be more effective.

Chemicals that are substrates for growth can be used at a variety of concentrations, and organisms that use these chemicals are generally found within the diffusion boundary. These organisms probably rely on substrates excreted by other nearby organisms within the diffusion boundary since, as discussed above, the flux of chemicals in the diffusion boundary is greater than in areas of mixing. Because flux of a substrate is often directly related to growth, the growth of an organism probably depends upon substrates provided by organisms within the same diffusion boundary.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Diffusion constrains chemically mediated interactions. Modes of chemically mediated interaction between organisms can be predicted given specific flow regimes and scales in streams. In general, interactions can be reciprocal within the diffusion boundary and are only unidirectional outside the diffusion boundary, unless the interacting organisms can move more rapidly than current. It is hoped the above discussion will convince ecologists that chemically mediated interactions are important in streams and will provide impetus for future research.

This paper reveals a number of areas in the chemical ecology of stream organisms where research is lacking. Nutrient competition is not well documented, substrate uptake kinetics for benthic algae are vastly under reported for organisms both inside and outside the diffusion boundary. Isotope experiments or nutrient removal experiments (Auer and Canale, 1982) would provide useful data on uptake kinetics. Although the importance of nutrient regeneration in streams has been recognized, rates of mineral flux have only been published for a handful of streams. Isotope dilution experiments as applied in oceanography (Laws, 1984) could be adapted for use in recirculating stream channels.

It may also be fruitful to look for allelochemicals that serve as growth cues in lotic systems exhibiting clear seasonal species succession. Such experiments would require coculture of primary producers, as was done by Keating (1977). Organic chemicals as attractants or repellents outside the diffusion boundary also may be important. Peckarsky (1980) and Peckarsky and Dodson (1980) describe a potentially useful approach using the behavioral response of species to imply such interaction. Extension of this approach would consist of gauging behavioral response to various chemical fractions of tissue extracts, eventually leading to isolation of the compounds that act as stimulus. Advanced analytical tools such as GC-MS and high-pressure liquid and other forms of chromatography available to chemical ecologists could aid in isolation of biologically active compounds.

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BEHAVIOR AND CHEMICAL DISGUISE OF CUCKOO ANT *Leptothorax kutteri* IN RELATION TO ITS HOST *Leptothorax acervorum*

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Abstract—Females of the obligately parasitic cuckoo ant, *Leptothorax kutteri*, a workerless inquiline, are among the only adult ants that can successfully invade ant societies and come to be accepted as a nestmate by the existing adult workers. This occurs even though the cuckoo ant is usually severely attacked by the *Leptothorax acervorum* workers of the colony that she is attempting to enter and parasitize. Through extensive ethogram studies of established parasites and parasitized and free-living *L. acervorum* workers and queens, we show that the *L. kutteri* queen grooms host queens at an exceptionally high frequency. Possibly associated with this behavior, the established parasite is never attacked by the *L. acervorum* workers or queens she exploits. We show that there is exceptional similarity between the cuticular hydrocarbons and especially the cuticular fatty acids of the parasitic females and her nestmate *L. acervorum* workers, compared with nonnestmate workers and queens. We suggest that this matching of cuticular compounds may be associated with the grooming of host queens by the parasite. This in turn suggests the possibility that fatty acids have a role in colony-specific nestmate recognition in these and other ants and that grooming may serve for the dissemination of such substances throughout the colony.

Key Words—Ants, Formicidae, Hymenoptera, *Leptothorax kutteri*, *Leptothorax acervorum*, nestmate recognition, ethograms, gas chromatography.

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INTRODUCTION

Many of the social parasites and guests of ants manipulate the lines of communication in colonies of their hosts, and therefore provide special opportunities to explore social and recognition signals in ants in general (Hölldobler, 1970, 1971; Kistner, 1979; Sudd and Franks, 1987). One of the socially parasitic species of ants that exploit other ants is *Leptothorax kutteri* (Buschinger, 1965). Together with other workerless inquilines and some slave-maker queens, this species is unusual in that adult females can enter foreign colonies of their host species and come to be accepted by the existing adults. By contrast, all interspecific slave-makers can only incorporate new workers into their colonies by capturing them in the form of larvae and pupae, before they have imprinted on their own colony odor. A key question, then, is how do cuckoo ants take over the recognition signals of their new host colonies?

In a recent paper, Allies et al. (1986) showed how a *L. kutteri* queen used secretions from its Dufour's gland to defend itself when entering foreign colonies of its host species *Leptothorax acervorum*. The parasitic queen is first violently attacked by the foreign host workers, but then she deploys her Dufour's secretion, which causes hostile host workers to attack one another rather than herself. In this paper, we extend this study to consider the behavior of the parasite within the nest of its host and examine both the chemistry and possible significance of the cuticular fatty acids and hydrocarbons of both the parasite and its host.

This is the first quantitative study of the behavior of a workerless inquiline ant within a host colony. We show that although the established parasites harass host queens and sometimes even eat their eggs, they are never attacked. This acceptance of an originally severely attacked parasite is particularly noteworthy, and we suggest that it may be associated with the extraordinary high rates at which the parasite grooms the host queens, which in turn may give rise to the considerable similarity in the cuticular compounds of the parasite and the host workers.

METHODS AND MATERIALS

Colonies of *Leptothorax acervorum* with and without their parasite *L. kutteri* were collected from dry pine woods in Blekinge, in southeastern Sweden in the summers of 1983 through 1986. Artificial nests were constructed by sandwiching a cardboard frame between two large microscope slides, to create a crevice (5.8 × 3.2 × 0.2 cm) within which the ants could reside. The nests were held within clear plastic containers with Fluon-coated walls and supplied with ample food and water. All the nests were kept in incubators following the

seasonal, temperature, and photoperiod-controlled culturing system of Buschinger (1973).

The behavior of all the ants within the nest could be observed on the stage of a dissection microscope either directly or through a video camera coupled to the microscope. Colonies were allowed 1 hr to acclimate before each recording period began. Recording sessions were of 60 min duration. A "focal animal" (Altmann, 1974) was selected at random from those present in the nest and continuously observed. This procedure was repeated 30 times for *L. kutteri* females, *L. acervorum* queens, and *L. acervorum* workers, making a total of 90 hr of direct observation of the different individuals.

To extend the sample sizes for the behaviors that most characteristically differed in frequency between the parasites and their hosts, video tapes were made using a time-lapse recorder and analyzed especially to record mutual grooming and feeding behavior among adult *L. kutteri* females and *L. acervorum* workers and queens. Other behaviors were not recorded from the tapes. The tape was examined using identical focal animal observation methods as used in direct observation. In this way, an additional 300 hr of observations were made. The initial observations of the study colony were made during its early summer phase in which all the egg laying took place. All the video observations were made for this and other colonies in their late summer phase when all egg laying had been completed. This seasonal change accounts for some of the relative differences in the rate of activity of the individuals in the nests. The colonies reached a peak of activity within the nest at the zenith of egg laying.

Chemistry. The workers from a colony were extracted as a group, as were the queens and parasites. Ants were extracted three times with 20 μl hexane (per extraction) for 10 min/extraction by simple soaking. The extracts were combined, concentrated to dryness under nitrogen, and reconstituted with hexane to a concentration of 1 ant/μl.

Assay by Capillary Column FIDGC. The extract was assayed on a Hewlett-Packard 5890A gas chromatograph with split–splitless injector and flame ionization detector. The GC column was a 25-m $\times$ 0.32-mm-ID cross-linked 5% phenyl methyl silicone WCOT (0.17 μm film thickness) fused silica capillary column (HP Ultra-2). The injector and detector were at 290°C. The carrier gas was helium at a 1.2 ml/min flow rate. Sample volumes of 0.8 μl and 2.5 μl were injected using a hot needle technique in the splitless mode with a splitless time of 1 min. The oven temperature program was 50°C for 2 min, 7°C/min to 200°C, 1°C/min to 280°C, and hold for 2 min. The FID output signal was plotted and quantified on a computing integrator (HP 3393A).

Analysis by Capillary Column GC-MS. The extracts were analyzed on a Finnigan OWA 1020 automated gas chromatograph–mass spectrometer (GC-MS) with INCOS software. The GC column was a 30-m $\times$ 0.32-mm-ID DB-5 WCOT (0.25 μm film) fused silica capillary column (Supelco). The split–

splitless injector, detector, and separator were held at 290°C. The carrier gas was helium at 28 cm/sec linear velocity. Sample volumes of 0.1–1.0 μl were injected using a hot-needle technique and a splitless time of 45 sec. The oven temperature program was 50°C for 2 min, 10°C/min to 280°C and hold for 30 min. The effluent of the GC column was introduced directly into the source of the MS. Spectra were obtained in the electron ionization mode with 70 eV ionization energy and manifold temperature of 80°C. The quadrupole mass analyzer was set to scan from 40 to 540 amu every 0.5 sec for a total of 6000 scans per sample. The tune of the MS was checked daily with FC43 and DFTPP to EPA standards. The fatty acids and *n*-alkanes were identified by coinjection of standards. Methyl-branched alkanes were identified by comparison of retention index and mass spectra with published data (Nelson, 1978; Nelson and Sukkestad, 1975; Nelson et al., 1972). Unsaturated hydrocarbons were identified as described elsewhere (Smith, 1989).

RESULTS

Behavioral Studies. The full comparative ethograms for *L. kutteri* females and for *L. acervorum* queens and workers show that in many of the behavioral categories there are striking similarities. However, there are also some large differences. It is clear that self-grooming is much more frequent in workers than in *L. kutteri* females or their own host queens, and workers also have much more frequent antennal contact with other workers. In the regurgitation of food (trophallaxis) *L. kutteri* is never seen to donate food to other ants but receives it from both host queens and workers (Tables 1–6). *L. acervorum* queens will occasionally give food to other queens and to *L. kutteri* but never to workers. Workers, by contrast, give food to all other individuals in the nest, including the parasite, but take it only from other workers. Interestingly, the rate at which *L. kutteri* is fed by *L. acervorum* queens is higher than the rate of mutual feeding among *L. acervorum* queens.

The largest difference in frequencies is in the grooming behavior. *L. kutteri* grooms both queens and workers at an extremely high rate: 24 times and 2.4 times higher rates, respectively, than such grooming of queens and workers by *L. acervorum* workers (Tables 1 and 2). Most remarkably, workers groom *L. kutteri* and other workers at a very similar but rather low rate. Workers groom queens at a much higher rate than they groom either other workers or *L. kutteri*. Host queens only groom one another and then only at a low rate. (Much of the difference in the corresponding values of grooming rates between focal and nonfocal animals is due to the comparative rarity of some individuals. Since there were 21 workers, four host queens and only one parasite female in the first study colony (Tables 1 and 2) the frequency of each behavior will tend to

TABLE 1.

Behavior	*L. kutteri*	*L. acervorum* Queen	*L. acervorum* Worker
1. Regurgitate food to *L. acervorum* worker	0	0	11
2. Regurgitate food to *L. acervorum* queen	0	0	6
3. Regurgitate food to *L. kutteri*		2	3
4. Receive food from *L. acervorum* worker	16	21	21
5. Receive food from *L. acervorum* queen	4	1	0
6. Receive food from *L. kutteri*		0	0
7. Feed at food source	0	0	0
8. Groom self	36	69	163
9. Groom/exchange food with larva	164	155	134
10. Groom *L. acervorum* worker	24	0	10
11. Groom *L. acervorum* queen	114	4	6
12. Groom *L. kutteri*		0	0
13. Groomed by *L. acervorum* worker	3	9	2
14. Groomed by *L. acervorum* queen	0	0	0
15. Groomed by *L. kutteri*		25	2
16. Antennal contact with *L. acervorum* worker	270	268	486
17. Antennal contact with *L. acervorum* queen	106	96	81
18. Antennal contact with *L. kutteri*		51	23
19. Move egg/larva/pupa	0	18	32
20. Lay egg	10	3	0
21. Interference with egg laying	26	0	0
22. Egg laying interfered with	0	2	0

be different if it was recorded directly as an act by a focal animal or indirectly as an act received from a nest mate.

L. kutteri groomed/exchanged food with larvae at a similar rate to its hosts (in the parasite this behavior consisted purely of obtaining food from the larvae,

TABLE 2. PARTIAL SUMMARY OF TABLE 1; COLONY S83.65 (COMPOSITION: 1 *L. kutteri*, 4 *L. acervorum* QUEENS, 21 WORKERS)

	Grooms			Groomed by			Feeds			Fed by		
Focal animal	L.k.	Q	W	L.k.	Q	W	L.k	Q	W	L.k.	Q	W
L. kutteri		144	24		0	3		0	0		4	16
L. acervorum queen	0	4	0	25	0	9	2	0	0	0	1	21
L. acervorum worker	0	6	10	2	0	2	3	6	11	0	0	21

TABLE 3. COLONY S83.65 (COMPOSITION: 1 *L. kutteri*, 4 *L. acervorum* QUEENS, 21 WORKERS)

	Grooms			Groomed by			Feeds			Fed by		
Focal animal	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W
L. kutteri		40	28		6	6		0	0		4	13
L. acervorum queen	1	4	1	14	3	3	1	0	1	0	0	4
L. acervorum worker	0	1	20	1	0	8	0	1	6	0	0	9

TABLE 4. COLONY S84.98 (COMPOSITION: 2 *L. kutteri*, 4 *L. acervorum* QUEENS, 16 WORKERS)

	Grooms			Groomed by			Feeds			Fed by		
Focal animal	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W
L. kutteri	0	21	16	0	0	2	0	0	0	0	4	5
L. acervorum queen	0	0	0	18	0	0	1	0	0	0	0	10
L. acervorum worker	0	0	8	2	0	1	2	3	6	0	0	9

TABLE 5. COLONY S84.94 (COMPOSITION: 4 *L. kutteri*, 0 *L. acervorum* QUEENS, 22 WORKERS)

	Grooms			Groomed by			Feeds			Fed by		
Focal animal	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W
L. kutteri	0		16	0		7	0		0	0		3
L. acervorum worker	1		7	5		3	0		0	0		2

TABLE 6. COLONY S83.95 (COMPOSITION: 0 *L. kutteri*, 3 *L. acervorum* QUEENS, 29 WORKERS)

	Grooms			Groomed by			Feeds			Fed by		
Focal animal	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W	L.k.	Q	W
L. acervorum queen		1	1		1	3		0	0		0	5
L. acervorum worker		3	16		0	9		5	7		5	7

which have digestive enzymes not present in adults), but unlike them did not carry out any brood care behavior. The *L. kutteri* queen laid eggs at a higher rate than its host. Perhaps, associated with this, *L. kutteri* interferes with the egg laying of its hosts. This consisted of the parasite approaching a queen during egg laying and aggressively grooming and biting it about the gaster. Such harassment would usually cause the queen to stop laying and run away. No host queens were seen interfering with the egg laying of others. On five occasions during the 30 hr of observation *L. kutteri* was seen to eat the eggs of its hosts. No egg predation was seen in *L. acervorum*.

The egg laying of two colonies with similar worker populations, one parasitized and one unparasitized, was monitored over an 11-day period. It can be seen from Figure 1 that *L. kutteri* has the fastest egg production rate (the parasite's eggs can be recognized by their small size), followed very closely by the egg production of the unparasitized colony. The parasitized colony has a much reduced rate of host egg production. It should be noted that both colonies had four *L. acervorum* queens, so the individual egg-laying rates of the hosts are almost certainly a fraction of that of the single parasite.

The tremendously high grooming rates by *L. kutteri* and other behavioral patterns among its nestmates (particularly of food exchange) were also seen in the other colonies studied (Tables 4–6). The main study colony (S8365) (see Tables 1–3) had one parasitic female, four host queens, and 21 workers. From

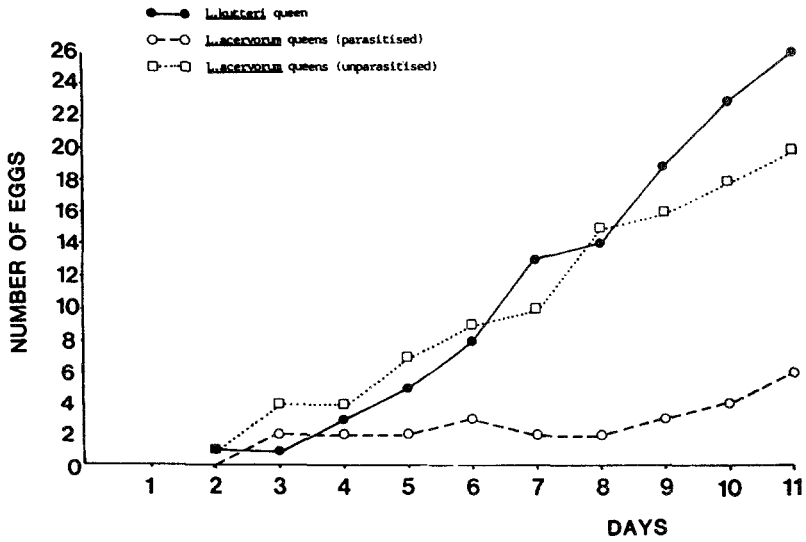

FIG. 1. The number of eggs laid by *L. acervorum* and *L. kutteri* queens in parasitized and unparasitized colonies.

a large selection of cultured colonies, we also examined behavior patterns in a colony with four host queens and two parasitic females, a colony with four parasitic females and no host queens, and one with no parasites and three host queens. These combinations of numbers of host queens and parasites were the same as when the colonies were censused immediately after collection from the field. All the study colonies were small with between 16 and 30 workers.

In all cases where host queens and parasites were present, the *L. kutteri* groomed the *L. acervorum* queens at extremely high frequencies per hour (Tables 2–4). In the absence of the host queens, *L. kutteri* grooms workers at a very high rate (Table 5), comparable to workers grooming their own queen in small unparasitized nests (Table 6). *L. kutteri* females never groom one another and were very rarely groomed by other colony members.

Since the *L. kutteri* females never do any work for the host colony, or even care for their own brood, we suggest that the parasite is not grooming host queens or workers to aid those individuals but to benefit herself. One such benefit would be if the parasite was able to gain and maintain the colony odor from her hosts, or so dilute their recognition system, that she can more easily remain disguised. This assertion is further supported by a preliminary experiment in which workers, host queens, and parasitic females were kept in isolation but given identical food to their original colony. After 10 days, each was returned to the original colony and the response of the host workers noted. Both *L. acervorum* queens and workers were reaccepted after a few seconds of grooming, as if they had only been out of the nest for a few minutes, whereas the *L. kutteri* was examined by a large number of workers and continuously groomed for several minutes. Apparently an *L. kutteri* female that has been unable to groom nestmates no longer closely resembles them in terms of recognition signals.

Chemical Results. The chromatograms of the cuticular compounds of these ants are complex (Figure 2), and this analysis was not designed to serve as an exhaustive examination of all classes of these components. Instead, to examine patterns among nestmates and between nonnestmates, the 18 hydrocarbons that could be quantified most accurately were selected. In addition, six fatty acids also could be identified and quantified (Tables 7 and 8). All four categories of ants (*L. kutteri*) parasitized workers from the nest as the *L. kutteri*, *L. acervorum* queens, and *L. acervorum* workers from the same unparasitized nest), had these 24 epicuticular compounds in common. To compare the four categories of individuals, we have used a standard similarity index:

$$SI = \Sigma\,(x - y) * (x - y)/E$$

where x and y are the normalized fractions of the same compound in the individuals being compared and E is the average amount of that compound for all four types of individual. The smaller the index, the greater the similarity. The

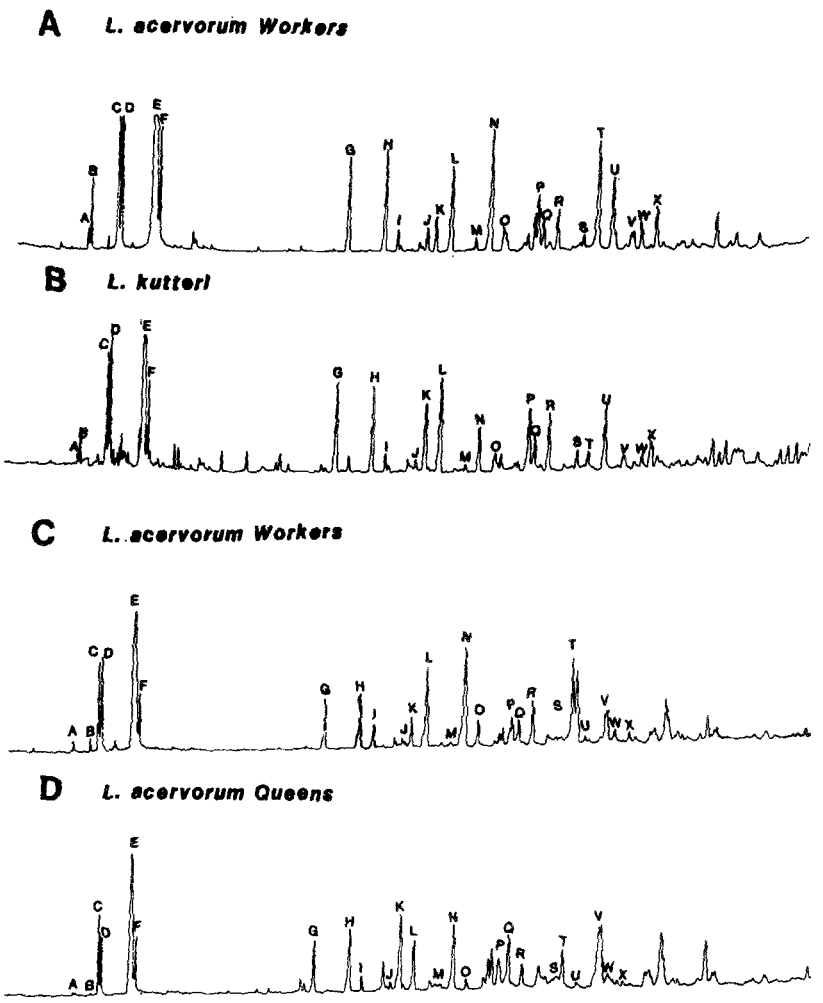

FIG. 2. Chromatographs for (A) *L. acervorum* workers and (B) *L. kutteri* from colony S8626, and (C) *L. acervorum* workers and (D) *L. acervorum* queens from colony S8573B. Peaks are lettered as in Tables 7 and 8 (see text for further details).

results were then summed over all classes of compound, i.e., either fatty acids or hydrocarbons. In this way a matrix of similarities has been constructed for all possible comparisons between individuals for both fatty acids and hydrocarbons. The pairings of different individuals could then be ranked from most similar to least similar and such ranking compared for fatty acids and hydrocarbons (Tables 9 and 10). The two rankings are significantly correlated (Spearman

TABLE 7. CUTICULAR ACIDS

Peak	Fatty acid	Normalized abundance: *L. acervorum* workers	Normalized abundance: *L. kutteri*
Colony S8626			
A	*cis*-9-Tetradecanoic	0.009	0.014
B	Tetradecanoic	0.024	0.016
C	*cis*-9-Hexadecenoic	0.145	0.138
D	Hexadecanoic	0.114	0.131
E	*cis*-9-Octadecenoic	0.647	0.626
F	Octadecanoic	0.060	0.075
Colony S8573B			*L. acervorum* queens
A	*cis*-9-Tetradecenoic	trace	trace
B	Tetradecanoic	0.016	0.011
C	*cis*-9-Hexadecenoic	0.0117	0.105
D	Hexadecanoic	0.1134	0.063
E	*cis*-9-Octadecenoic	0.705	0.754
F	Octadecanoic	0.049	0.067

correlation coefficient $rs = 0.88$ $p = 0.05$). Most intriguingly, the parasite and the parasitized workers are the most similar in terms of fatty acids and second most similar in terms of hydrocarbons. For both classifications, the parasite and the parasitized workers were more similar to one another than were the conspecific *L. acervorum* queens and workers from the unparasitized control colony.

DISCUSSION

The cuckoo ant, *L. kutteri* is almost certainly a sibling species of its host *L. acervorum* (Buschinger, 1965). *L. kutteri* is extremely similar in appearance to *L. acervorum* queens. However, the cuckoo ants are smaller than their host queens, being similar in size to host workers. Nevertheless, the parasites behave in a unique way. They are true social parasites, producing no workers of their own and showing no brood care. They rely on host workers (and queens) to supply them with food and to rear their purely sexual offspring. In addition, both to gain extra food directly and possibly to subvert the host colony into rearing more of her progeny rather than their own, the cuckoo ant predates the eggs of host queens and interferes with egg laying. Despite her direct and indi-

TABLE 8. SELECTED CUTICULAR HYDROCARBONS

Peak	Hydrocarbon	Normalized abundance	
		L. acervorum workers	*L. kutteri*
Colony S8626			
G	C27:0	0.080	0.111
H	3-Me-C27:0	0.092	0.094
I	C28:0	0.017	0.018
J	C29:2	0.020	0.016
K	9-C29:1	0.030	0.096
L	C29:0	0.085	0.128
M	11-Me-C29:0	0.012	0.010
N	3-Me-C29:0	0.144	0.049
O	C30:0	0.023	0.039
P	C31:2	0.065	0.100
Q	9-C31:1	0.031	0.051
R	C31:0	0.039	0.071
S	UNK	0.013	0.024
T	3-Me-C31:0	0.144	0.020
U	UNK	0.089	0.095
V	C33:2	0.038	0.024
W	UNK	0.030	0.016
X	11-Me-C33:0	0.048	0.039
			L. acervorum queens
Colony S8373B			
G	C27:0	0.057	0.058
H	3-Me-C27:0	0.066	0.083
I	C28:0	0.026	0.018
J	C29:2	0.014	0.011
K	9-C29:1	0.037	0.114
L	C29:0	0.122	0.067
M	11-Me-C29:0	0.005	0.003
N	3-Me-C29:0	0.181	0.098
O	C30:0	0.032	0.015
P	C31:2	0.061	0.073
Q	9-C31:1	0.038	0.090
R	C31:0	0.061	0.041
S	UNK	0.009	0.007
T	3-Me-C31:0	0.151	0.054
U	UNK	0.011	0.008
V	C33:2	0.084	0.221
W	UNK	0.027	0.030
X	11-Me-C33:0	0.019	0.010

TABLE 9. SIMILARITY MATRIX FOR EPICUTICULAR FATTY ACIDS

	L. kutteri	Parasitized *L. acervorum* workers	Free-living *L. acervorum* queens	Free-living *L. acervorum* workers
L. kutteri		0.123	0.320	0.2314
Parasitized *L. acervorum* workers			0.2694	0.1647
Free-Living *L. acervorum* queens				0.184
Free-living *L. acervorum* workers				

TABLE 10. SIMILARITY MATRIX FOR EPICUTICULAR HYDROCARBONS

	L. kutteri	Parasitized *L. acervorum* workers	Free-living *L. acervorum* queens	Free-living *L. acervorum* workers
L. kutteri		0.638	0.924	0.821
Parasitized *L. acervorum* workers			0.9582	0.491
Free-Living *L. acervorum* queens				0.792
Free-Living *L. acervorum* workers				

rect interference with the host colony and its members, the *L. kutteri* female, once established, is never attacked.

Our analysis of epicuticular compounds points to one reason why the cuckoo ant may remain undetected by host workers—she is extremely similar to the workers in the colony she is parasitizing. A number of authors (Bonazita-Cougourdan et al., 1987; see also Blum, 1987, for review) have suggested that cuticular hydrocarbons may be the basis of nestmate recognition, although no bioassays have been devised yet to link these compounds with the recognition behavior. Our results suggest that fatty acids also should be included in the design of such bioassays. Indeed, it is even possible that fatty acids play a more important role than cuticular hydrocarbons in nestmate recognition in ants.

The cuticular hydrocarbons of workers from different nests were more similar than the within-nest comparison, even though such nonnestmate workers will attack one another. Clearly another set of discriminators, e.g., the fatty acids, must also be involved. One possible explanation why the parasite and the parasitized workers were more similar in both fatty acids and hydrocarbons than are related queens and workers in pure nests, is that the workers may themselves be obtaining their odors by grooming the queens. This similarity then may be due to both parasites and workers deriving their odors with similarly biased sampling from the same source. Thus the derivatives are more similar to one another than they are to the source itself.

Another possibility, which we cannot dismiss completely at this stage, is that the parasite is changing and or diluting the nestmate recognition signals throughout the parasitized colony by grooming the host queens. However, since some species of ants are known to have an inherited component to their nestmate recognition systems (Mintzer, 1982), this seems unlikely. Many types of social contact may cause cuticular compounds to be picked up by one or both of the interacting insects (see Vander Meer and Wojcik, 1982), and contact with the brood may also be important. However, since the interspecific parasite *L. kutteri* is more similar to the workers in the colony she is exploiting than are related conspecific queens and workers in the same unparasitized nest, this does emphasize the possible importance of the highly active grooming behavior of the cuckoo ant.

High rates of grooming of their hosts by the guests and parasites of other ants have been recorded anecdotally many times before, e.g., licking of the host queen by the parasite *Teleutomyrmex* (Wilson, 1971). The shampoo ant *Leptothorax emersoni*, a xenobiont, as its name implies, voraciously licks its hosts *Myrmica canadensis* (Wheeler, 1910). [These species are now recognized as *Formicoxenus provancheri* and *Myrmica incompleta*, respectively (Francoeur et al., 1985).] A number of convergently evolved staphylinid beetle guests of army ants adopt unique and highly ritualized grooming positions when they vigorously lick their hosts (Akre and Torgerson, 1968). Rettenmeyer (1961) suggested that such grooming specifically enabled the beetles to pick up the colony-specific odor of their host colony.

We tentatively suggest, therefore, on the basis of these and our own observations of the behavior and chemistry of *L. kutteri* cuckoo ants in relation to their hosts, that grooming plays a key role in the dissemination of recognition signals within nests and that cuticular fatty acids may be previously unrecognized key markers.

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LEAF PHENOLIC INHIBITION OF GYPSY MOTH NUCLEAR POLYHEDROSIS VIRUS
Role of Polyhedral Inclusion Body Aggregation

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Abstract—Bioassays with nuclear polyhedrosis virus (NPV) administered to gypsy moth larvae on leaf disks from various tree species reveal strong viral inhibition by some tree species. Phenolic extracts from inhibitory tree leaves cause virus polyhedral inclusion bodies (PIBs) to form large aggregations. However, aggregated PIBs treated with leaf extracts and administered to larvae on laboratory diet (without phenolics) retain virulence. Our results suggest that leaf phenolics, especially hydrolyzable tannins, inhibit NPV infection, but may not act via aggregations formed in the foregut.

Key Words—Gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, tannins, nuclear polyhedrosis virus, phenolics.

INTRODUCTION

The susceptibility of gypsy moth *Lymantria dispar* (Lepidoptera, Lymantridae, larvae to the gypsy moth nuclear polyhedrosis virus (GMNPV) can be influenced by the foliage of the plant species on which the viral polyhedral inclusion bodies (PIBs) are consumed (Keating and Yendol, 1987). Differences in larval susceptibility may be the result of the interactions between the gypsy moth virus

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and various foliage chemicals (Keating et al., 1988). Specifically, previous work has shown that decreases in larval mortality are strongly correlated with increasing levels of hydrolyzable tannin in natural and artificial diets (Keating et al., 1988; Keating and Yendol, 1988).

The GMNPV plays a conspicuous role in gypsy moth population dynamics (USDA, 1981). For example, GMNPV epizootics are closely associated with outbreak collapse. Because leaf phenolics, especially hydrolyzable tannins, can reduce gypsy moth growth and fecundity (Rossiter et al., 1988), the possibility of enhanced resistance to disease acquired from the same chemicals needs to be explored before we can understand the host plant's net influence on gypsy moth population dynamics.

Tannins are polyphenolic compounds capable of binding to proteins and reducing the activity of many enzymes (Swain, 1979). While the ecological significance of these phenolics is in dispute, tannins can be strongly inhibitory of wide range of microorganisms including viruses, bacteria, and fungi and thus may protect organisms from pathogens (Swain, 1979). The possible mechanisms for antimicrobial activity are several but could certainly include the formation of complexes between tannins and microbial proteins (Cadman, 1960).

Virions of GMNPV are occluded in a paracrystalline matrix composed of polyhedrin protein (USDA, 1981). The polyhedral inclusion bodies (PIBs) are ingested and pass first through the larval foregut where pH levels (pH 4.0–8.0) may be adequate for the occurrence of tannin–polyhedrin binding (Schultz and Lechowicz, 1986). When PIBs enter the midgut, the imbedded virions are released when the protein matrix dissolves because of alkaline pH levels and protease activity (McCarthy and DiCapua, 1979). Since virions must be released from the inclusion bodies before infection can occur, we hypothesized that tannin–polyhedrin complexes formed in the foregut may reduce larval mortality by inhibiting PIB dissolution and virion release.

The goals of this study were to confirm the positive correlations between foliage tannin content or protein-binding capacity and reductions in larval susceptibility to virus, to examine the possibility that foliage tannins might form complexes with PIB proteins of the GMNPV, and to determine whether tannin binding of PIBs explains viral inhibition on foliage.

METHODS AND MATERIALS

Bioassays with Virus. The susceptibility of gypsy moth larvae to the GMNPPV when the virus was consumed on different host plants was examined using methods previously described by Keating and Yendol (1987). In brief, larvae were reared through second instar on an artificial diet, removed from food for 24 hr upon molting to third instar, and then allowed 24 hr to consume

a single 10-mm-diameter disk of host-plant foliage treated with 60,000 PIBs/disk. Larvae not consuming a complete dose were discarded. Control larvae received virus-free leaf disks to test for any mortality resulting from diet-induced stress. Following inoculation, larvae were returned to artificial diet and held individually for a 17-day incubation period during which mortality resulting from virus was recorded daily. Since larvae were treated identically before and after inoculation, differences in mortality among larvae inoculated with the same viral dose but on different diets can be attributed to the effect of foliage on virus or the infection process. Host-plant species were red oak (*Quercus rubra* L.), black oak (*Q. velutina* Lam.), white oak (*Q. alba* L.), quaking aspen (*Populus tremuloides* Michx), bigtoothed aspen (*P. grandidentata* Michx.), and red maple (*Acer rubrum* L.). All but the latter are highly favored, suitable hosts for the gypsy moth; red maple is a common codominant with preferred hosts (USDA, 1981).

The significance of differences in mortality among larval groups inoculated on different host-plant species was assessed using an ANOVA of the arcsin-transformed percent mortality in each replication (Steel and Torrie, 1980). Treatments on each host-plant species were replicated four times with approximately 50 larvae in each replicate. Significant differences in mortality were determined using Duncan's new multiple-range test (Steel and Torrie, 1980).

Chemical Analysis of Leaf Material. Concurrent with the virus bioassay, additional leaf samples were collected for chemical analysis from the same trees used in the bioassay, and the foliage was treated in the same manner as bioassay foliage prior to any analysis. Three samples from each of six host-plant species were frozen with liquid nitrogen, ground into small fragments, freeze-dried, and then further ground to a fine powder in a cyclone mill. Approximately 400 mg of powder was washed twice with diethyl ether and extracted in 20 ml of 70% acetone. The acetone was removed by evaporation under reduced pressure, and distilled water was added to the aqueous extracts to bring the final extract volume to 10 ml.

At the same time, 10 leaves were collected from each tree, weighed and dried at 104°C for 48 hr. They were reweighed and used to calculate percent dry weight for each tissue used in this study. Mean dry weight/wet weight ratios were used to calculate the dry mass of disks used in bioassays (above); for each tree, 30 disks actually used in bioassays were weighed fresh for this calculation. We could then calculate the actual fraction of leaf disk fresh weight that comprised the various phenolic constituents, and thus the phenolic dose consumed in each disk with virus.

The hydrolyzable tannin content of the aqueous extracts was estimated as galloyl esters using a potassium iodate reagent (Bate-Smith, 1977) under carefully timed conditions modified to assure quantitative results (Schultz and Baldwin, 1982). Condensed tannins were estimated as leucoanthocyanins (Bate-

Smith, 1975) and the protein-binding capacity of extracts ("astringency") was determined using hemoglobin as a substrate and tannic acid as a standard (Schultz and Baldwin, 1982; Schultz et al., 1981). Levels of hydrolyzable tannins and astringency are expressed as percent dry weight tannic acid (Sigma, Inc., batch T-0125) equivalents (%TAE). Condensed tannin levels are expressed as percent dry weight wattle tannin equivalents (%WTE) from a purchased standard (Leon Monnier, Inc.). Percent dry weight equivalents were converted to fresh weight values using average dry weight/fresh weight ratios derived from samples of 10 leaves of each foliage type (above). The total amount of equivalents in the bioassay leaf disks was then calculated using the average fresh weight of the disks.

Differences between host-plant species in tannin content and binding capacity of the foliage were determined using Duncan's new multiple-range test following ANOVA of the data. The relationship between leaf chemistry and larval susceptibility to virus was assessed by simple regression of weighted (1/variance) average larval mortality following virus treatment on each host-plant species on tannin contents, and binding capacity of the leaf disks from the same trees (Steel and Torrie, 1980).

PIB Aggregation Assay. Preliminary work indicated that the addition of aqueous leaf extracts to suspensions of GMNPV PIBs resulted in the formation of large aggregates of PIBs. To quantify this observation, 0.925 ml aqueous leaf extract from the above chemical analysis as combined with 0.075 ml from a 1.5×10^8 PIBs/ml suspension and gently stirred for 15 min. A 15-min interval represents the median residence time for leaf material and PIBs in the larval foregut (Keating and Schultz, unpublished observations). A sample was drawn from the mixture, added to a hemacytometer, and observed at 400× magnification. PIBs were considered unaggregated if they appeared to be more than one PIB diameter from the nearest PIB and/or if they appeared to have independent brownian movement. The number of unaggregated PIBs observed in a $0.2 \times 0.2 \times 0.1$-mm field was recorded, and the average number of unaggregated PIBs in 10 fields was used as an inverse measure of the aggregating capacity of the leaf extract sample. PIBs were added to 0.925 ml of distilled water to determine the number of unaggregated PIBs in an extract-free suspension. The experiment was repeated using 1 : 10 dilutions of each leaf sample to determine the effect of changing foliage chemical concentrations on PIB aggregation.

Finally, the PIB aggregation assay was repeated using tannic acid solutions (Sigma, Inc., batch T-0125) of four different concentrations prepared in 0.05 M MES buffers to determine the aggregating capacity of this commercial hydrolyzable tannin. The pH of the tannin–buffer solutions was adjusted to pH 5.0 to approximate pH levels found in the foregut when filled with macerated leaf tissue. Tannin concentrations were adjusted so that the addition of 0.075 ml of PIB suspension to 0.925 ml of tannic acid solution resulted in final tannin concentrations of 1.0, 0.2, 0.04, and 0.008%. The average number of unaggre-

gated PIBs was again determined for 10 fields per replicate and each tannin-concentration treatment was fully replicated three times.

Differences in the PIB aggregating capacity between extracts of different host-plant species were determined using Duncan's new multiple-range test following ANOVA of the data. The relationship between leaf chemistry and PIB aggregating capacity and between aggregating capacity and larval mortality was assessed with GLM (SAS Institute) and linear regression analysis (Steel and Torrie, 1980).

Bioassays with Aggregated PIBs. To determine whether PIBs aggregated by tannins had lost infectivity, viral bioassays were performed as described above. However, viral PIBs were administered on laboratory diet plugs instead of leaf disks (Keating et al., 1988). A new set of extracts from quaking aspen (*Populus tremuloides* Michx.), red oak (*Quercus rubra* L.), and pitch pine (*Pinus rigida* P. Mill) leaves was prepared as described above, but with 1 g of leaf powder in 10 ml of 70% acetone. Pitch pine was selected because of reports that late-instar larvae feeding on it appeared relatively resistant to viral mortality (Rossiter, 1987).

Acetone was removed by evaporation at reduced pressure to produce aqueous extracts with phenolic concentrations approximating those in spring leaf tissue (Keating and Schultz, unpublished data; Rossiter et al., 1988). Thus, these extracts were about 10 times more concentrated than the undiluted extracts used in the PIB aggregation assay described above. Dilutions (1:5) of these highly concentrated extracts were also used to investigate changes in GMNPV efficacy with changes in leaf chemical concentration. PIBs, 4×10^6, were added to 1.0 ml of each extract and stirred for 30 mins. After extract treatment, PIBs were resuspended in sterile distilled water. Microscopic examination confirmed that extract-treated PIBs remained aggregated following resuspension. The infectivity of the PIBs was assessed by feeding third-instar larvae a single artificial diet plug treated with 2.0 μl of 4.0×10^5 PIB/ml suspensions of virus. Thus, individual larvae received approximately 800 PIBs/larva of extract-treated or distilled water-treated virus. Control larvae received diet plugs treated with virus-free distilled water. All other bioassay conditions were as described above.

Leaf extracts were assayed for hydrolyzable and condensed tannin contents, protein binding, and ability to aggregate PIBs as described above. Significance of variation in mortality among extract types and phenolic concentrations was assessed using ANOVA (SAS Institute).

RESULTS

Bioassays with Virus. The susceptibility of larvae to GMNPV was strongly affected by the type of foliage consumed in conjunction with the virus. Larvae fed virus on red maple and red or black oak leaf disks showed significantly

lower mortality when compared with larvae fed either species of aspen; mortality on white oak was intermediate (Table 1). Mortality among control larvae was less than 2% for each host-plant species.

Chemical Analysis of Leaf Material. Foliage from different host-plant species also differed significantly in hydrolyzable and condensed tannin content and protein binding capacity. Leaf disks from foliage collected from the black and red oak trees provided significantly more hydrolyzable tannin per disk (in TAE) than did disks from the other trees, and white oak and red maple foliage contained significantly more hydrolyzable tannin per disk than did quaking or bigtooth aspen (Table 1). Disks from red and black oak contained significantly greater protein binding capacity than did disks cut from white oak, red maple, quaking aspen, and bigtooth aspen (Table 1). Condensed tannin estimates were highest for the two aspen species, intermediate for red maple and black oak, and lowest for red and white oak.

A significant negative relationship was found between larval mortality and hydrolyzable tannin content of leaf disks ($P \leq 0.02$, $r^2 = 0.79$). The relationship between hemoglobin binding and mortality was negative but weak ($P = 0.07$). No significant relationship was found between mortality and condensed tannin content ($P = 0.31$).

PIB Aggregation Assay. The capacity of foliage extract to promote the formation of PIB aggregates was clearly dependent on the type of foliage used and the concentration of the extracts (Table 2). When PIBs were treated with undiluted extracts (400 mg powder in 10 ml H_2O), all extracts showed some

TABLE 1. MEAN (± SD) MORTALITY FOR LARVAE INOCULATED WITH 60,000 PIBs/LARVA ON LEAF DISKS OF SIX HOST-PLANT SPECIES, AND MEAN (± SD) VALUES FOR TANNIN CONTENT AND PROTEIN BINDING CAPACITY OF LEAF DISKS FROM THE SAME SPECIES[a]

Host-plant species	Mortality (%)	Hydrolyzable tannin (mg/disk)	Condensed tannin (mg/disk)	Protein binding (mg/disk)
Red maple	44.7 (14.3)a	0.44 (0.04)b	0.2444 (0.033)a	0.12 (0.01)b
Black oak	49.5 (7.2)a	0.86 (0.23)a	0.2137 (0.074)a	0.22 (0.01)a
Red oak	52.0 (16.1)a	0.74 (0.14)a	0.0076 (0.007)b	0.23 (0.02)a
White oak	79.0 (2.0)b	0.52 (0.02)b	0.0136 (0.004)b	0.13 (0.01)b
Quaking aspen	91.5 (4.1)c	0.11 (0.01)c	0.6368 (0.039)c	0.13 (0.01)b
Bigtooth aspen	96.0 (2.8)c	0.09 (0.01)c	0.5481 (0.050)c	0.11 (0.02)b

[a] Chemical units are mg tannic acid or wattle tannin per leaf disc. Mortality data were square root arcsine transformed for analysis; untransformed values are reported here. Values followed by the same letter are not significantly different ($P > 0.05$).

TABLE 2. MEAN (± SD) NUMBER OF UNAGGREGATED PIBS REMAINING IN 0.04 mm^2 HEMACYTOMETER FIELD AFTER TREATMENT FOR 15 MINUTES IN HOST-PLANT FOLIAGE EXTRACTS[a]

Host-plant species	Unaggregated PIBs	
	Condensed extract	Dilute extract
Red maple	7.5 (0.91)a	19.5 (1.88)b
Black oak	11.3 (2.25)ab	20.0 (3.92)b
Red oak	6.3 (0.69)a	10.8 (2.31)a
White oak	8.3 (1.68)a	28.1 (1.80)c
Quaking aspen	17.2 (5.01)c	44.3 (0.65)d
Bigtooth aspen	15.7 (4.36)bc	44.6 (1.74)d
Control (no extract)	46.6 (3.07)d	

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

PIB aggregating capacity. Significantly fewer unaggregated PIBs were found following treatment with red oak, red maple, and white oak extracts than with the undiluted quaking and bigtooth aspen extracts (Table 2). Differences were even greater following a 1 : 10 dilution of all extracts. Dilute red oak extracts appeared to have a significantly greater PIB aggregating capacity than did the extracts from any other host-plant species (Table 2). Diluted black oak and red maple extracts had significantly greater aggregating capacities than did dilute extracts from white oak, quaking aspen and bigtooth aspen. Finally, aggregation was significantly greater in dilute white oak extract than in dilute quaking or bigtooth aspen, which were indistinguishable from controls.

PIB aggregation was greater in extracts having higher hydrolyzable tannin contents and higher protein binding capacity. The number of free (unaggregated) PIBs after treatment in extracts was negatively correlated with hydrolyzable tannin contents of both undilute ($r^2 = 0.40$, $P < 0.005$) and dilute ($r^2 = 0.54$, $P < 0.001$) extracts (Figure 1). The protein binding capacity of the extracts also was negatively correlated (undilute $r^2 = 0.25$, $P < 0.03$; dilute $r^2 = 0.46$, $P = 0.002$), with the number of unaggregated PIBs found at both foliage extract concentrations. However, the condensed tannin content of extracts was negatively correlated with aggregation ($r^2 = 0.79$, $P = 0.03$ dilute; $r^2 = 0.87$, $P = 0.006$ concentrated).

Larval mortality due to virus was less when larvae received virus on foliage with strong aggregating capacity (Table 1). Average larval mortality following consumption of virus on host-plant foliage was negatively correlated with PIB aggregation in undilute extracts, although statistical significance was marginal ($P = 0.07$) despite a relatively large r value ($r = 0.799$). The marginal signif-

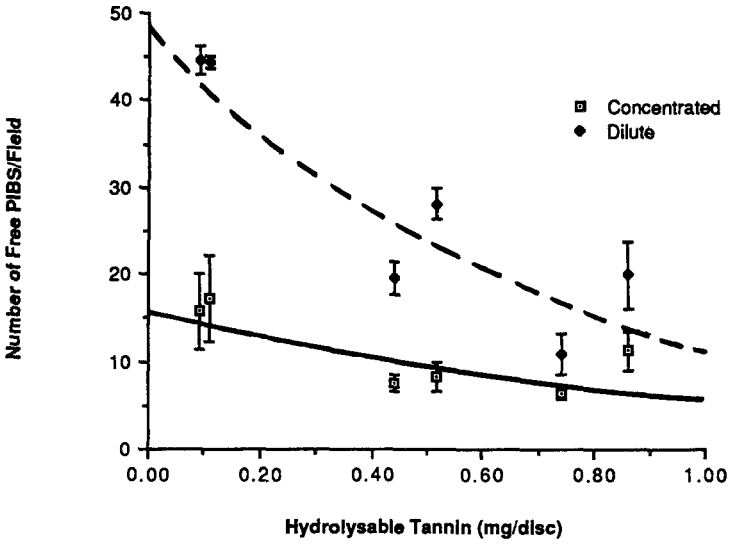

FIG. 1. Numbers of GMNPV PIBs left unaggregated ("free") per standard microscope field as a function of hydrolyzable tannin concentrations in disks cut from foliage of six tree species (solid line, see Table 2). Bars represent 1 SD. Regression for concentrated extracts: $Y = 48.32 \times 10^{-0.63} X$, $R^2 = 0.46$. Regression for dilute (1:5) extracts: $Y = 15.46 \times 10^{-0.38}$, $R^2 = 0.71$.

icance may be a result of the saturation of the aggregating capacity of oak and maple extract; a few PIBs always remained unaggregated. However, there was a significant positive correlation between larval mortality in the leaf disk bioassay and the average number of unaggregated PIBs following treatment with dilute extracts of the same species ($r = 0.929$, $P = 0.008$). A linear regression of this relationship yielded the equation: $Y = 1.51X + 26.6$ with an r^2 of 0.857, where Y is mortality and X is the number of unaggregated PIBs. Larval mortality was greatest on quaking and bigtooth aspen leaf material and dilute extracts of this foliage showed little or no PIB aggregating capacity compared with the oaks or red maple.

Observations of PIBs treated with tannic acid solutions demonstrated that hydrolyzable tannins can cause PIBs to aggregate. Aggregating activity was strongly dependent on tannin concentration, with few unaggregated PIBs remaining after treatment with 1.0% tannic acid (Figure 2).

Bioassays with Aggregated PIBs. PIBs treated directly with concentrated (3.33 g powder/10 ml water) or dilute (0.66 g powder/10 ml water) leaf extracts and aggregated prior to inoculation yielded larval mortalities indistinguishable

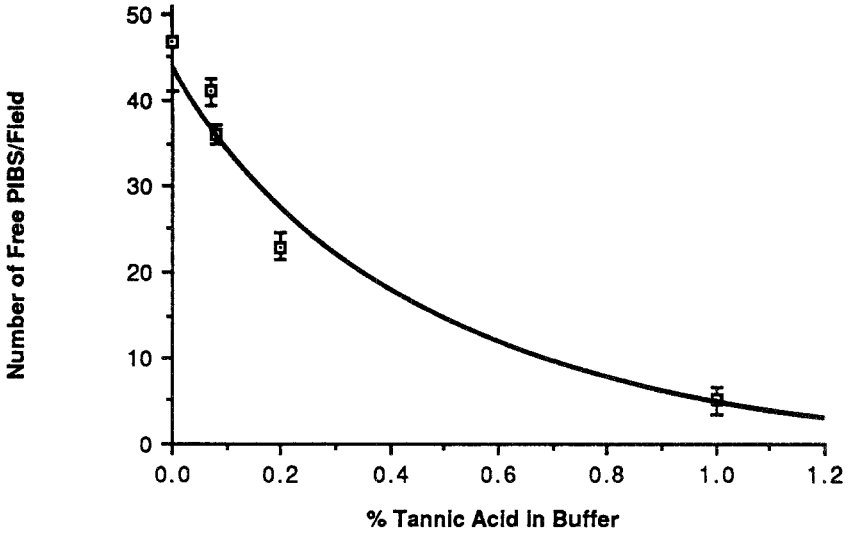

FIG. 2. Numbers of GMNPV PIBs left unaggregated ("free") per standard microscope field at increasing concentrations (w/v) of purified tannic acid in 0.05 M MES-buffered water medium. Bars represent 1 SD. Regression: $Y = 43.43 \times 10^{-0.95} X$, $R^2 = 0.98$.

($P > 0.05$) from untreated controls when administered on lab diet (Table 3). Control and treatment mortalities were somewhat higher in this experiment and chemical measures were somewhat lower than in previous trials (Table 4). However, these extracts were shown to aggregate PIBs effectively (Table 3).

TABLE 3. MEAN (SD) PERCENT MORTALITIES OF LARVAE INOCULATED WITH 800 PIBS PRETREATED AND AGGREGATED WITH EXTRACTS OF THREE TREE SPECIES, AND MEAN (SD) NUMBER OF UNAGGREGATED PIBS IN EACH PRETREATMENT[a]

Host-plant species	Mortality (%)		
	Concentrated extract	Dilute extract	Unaggregated PIBs
Red oak	91.25 (2.50)	93.75 (2.76)	8.53 (0.48)
Pitch pine	93.00 (1.47)	91.25 (2.76)	9.30 (1.49)
Quaking aspen	90.62 (2.47)	92.48 (4.24)	9.43 (0.58)
Control (lab diet)	91.80 (2.49)	88.58 (3.04)	46.60 (3.07)[a]

[a] Value followed by letter is significantly different from the rest in the same column ($P < 0.05$).

TABLE 4. PHENOLIC CONTENTS OF LEAVES EXTRACTED AND USED IN PIB AGGREGATION PRETREATMENT

Host-plant species	Hydrolyzable tannins (% TAE)[a]	Total phenolics (% TAE)	Protein binding (% TAE)
Red oak	22.33	3.82	4.16
Pitch pine	13.41	1.66	2.22
Quaking aspen	15.18	3.18	3.05

[a] TAE = tannic acid equivalents as % fresh weight.

DISCUSSION

The susceptibility of gypsy moth larvae to the GMNPV was significantly affected by the type of foliage consumed in conjunction with the virus. Larvae fed virus-treated foliage from oak and maple species had lower mortalities than larvae fed virus-treated foliage from aspen species. Decreased susceptibility to the virus was negatively correlated with the hydrolyzable tannin content of the leaf disks, but not significantly correlated with foliage hemoglobin binding capacity. These results are essentially consistent with previous observations, although past studies have found significant negative correlations between mortality and hemoglobin binding (Keating and Yendol, 1987; Keating et al., 1988).

When treated with phenolic extracts from foliage, PIBs formed large aggregates. This strongly suggests that compounds in the extracts were able to bind to the proteinaceous PIBs. The resulting PIB agglutination was dependent on both extract concentration and on the extract source. Extracts from oak and maple foliage were clearly more effective at promoting the formation of PIB aggregations than were extracts from aspen foliage. The ability of commercial tannic acid to promote aggregation suggests that it is indeed the tannins in these extracts that are responsible for the aggregation of PIBs.

The aggregating capacity of the extracts was strongly correlated with extract hydrolyzable tannin content and somewhat correlated with hemoglobin binding capacity. In general, fewer unaggregated PIBs remained following treatment with extracts containing higher tannic acid equivalents (TAE). Red maple extracts were an exception; maple extracts were as effective as oak extracts at causing PIB aggregation despite lower hydrolyzable tannin concentrations and much lower hemoglobin binding capacities. Red maple tannins may have a greater affinity for polyhedrin than hemoglobin, and the lower affinity of red maple tannins for hemoglobin may account for the absence of a significant cor-

relation between mortality and hemoglobin binding. In general, it appears that interactions between tannin and hemoglobin may be a poor model for interactions between phenolics and viral proteins.

Surprisingly, the correlation between aggregation of PIBs and condensed tannins was negative. We had expected condensed tannins to bind to PIB proteins as strongly or more strongly than hydrolyzable tannins. Wattle tannin, a mixture of condensed tannins from *Acacia* sp., exhibits weak but significant viral inhibition in lab diet bioassays (Keating and Yendol, 1988). However, leaf condensed tannins generally do not predict larval mortality to NPV when administered on leaf disks (Keating et al., 1988). As before, we observed strong negative correlations between hydrolyzable and condensed tannin measures within and among the tree species studied (Rossiter et al., 1988; Schultz, unpublished data). We suggest that this correlation creates an artificial negative relationship between condensed tannin measures and PIB aggregation. Condensed tannin measures in aspen leaves may actually reflect concentrations of nonbinding monomeric flavonoids, as indicated by the weak hemoglobin binding of aspen extracts (Table 1). Condensed tannins appear to play a minor role, if any, in gypsy moth viral inhibition.

We had expected larval susceptibility to virus on different host-plant species to be related to the ability of foliage extracts to promote PIB aggregation. In general, foliage types that reduced mortality most in leaf disk bioassays also yielded extracts that aggregated PIBs most strongly, and these mortality patterns parallel previous bioassay results using these tree species (Keating and Yendol, 1987).

However, PIBs recovered from extract aggregations were as lethal as normal unaggregated PIBs (Table 3). Because these extracts clearly formed PIB aggregations prior to consumption by larvae, our results suggest that binding tannins to PIBs and PIB aggregation alone did not inhibit viral infection in these experiments. Hydrogen-bonded tannin–polyhedrin complexes may have quickly dissociated in the midgut because of alkaline or detergent conditions (Martin et al., 1985). While the frass was not examined for intact PIBs, indirect evidence suggests that PIBs dissolved and released virions at the same rate whether treated with extracts or water, because mortality was similar among larvae fed extract- and water-treated PIBs.

There may be several explanations for the discrepancy between PIB aggregation–mortality correlations and the absence of inhibition of the virus following direct treatment with extracts. First, leaves (and leaf disks) are more strongly buffered than is lab diet, and larval midgut pH levels are lower when larvae consume well-buffered plant material (Schultz and Lechowicz, 1986). This could permit ingested aggregations to remain intact longer than in our experiments with unbuffered lab diet. Previous work (Keating et al., 1988, and unpublished) showed that the presence of phenolics in the diet, especially tannins, inhibits

GMNPV activity regardless of buffering conditions, but that the impact was increased when acid buffers were added to the diet.

Second, tannins bound to PIBs may have had greater affinity for lab diet components (such as agar or wheat germ) than for viral proteins, and PIBs may have been released by competitive interaction of the tannin with lab diet components.

Third, the concentrations of phenolics actually accompanying aggregated PIBs into the insect in these experiments were much less than when leaves are consumed. If tannin–PIB interactions are dynamic, higher concentrations of tannins may be required in the digestive tract to keep PIBs and tannins complexed, especially if competitive substrates are available or pH conditions are unfavorable for tannin–polyhedrin binding.

Fourth, leaf phenolics may undergo oxidation in the midgut to form quinones (Felton et al., 1987). These can bind covalently with virions, inactivating them (Cadman, 1960). Perhaps such complexes slow PIB dissolution and deactivate virions that are not exposed to phenolics until released in the midgut.

Finally, ingested tannins could bind to or otherwise modify the larva's peritrophic membrane, a proteinaceous network through which virions must pass to enter the midgut epithelium (see Feeny, 1970; Richards and Richards, 1970). All of these mechanisms would require high tannin concentrations in the gut itself.

It is clear that leaves of various tree species inhibit GMNPV differentially, and phenolics (especially tannins) are implicated as causal factors. However, inhibition seems to be dependent on conditions and chemical concentrations in the larval midgut, and the precise mechanism remains unknown. These results corroborate field observations that larval mortality from virus varies among host-plant species (USDA, 1981), although further work is required to determine whether host plant–virus interactions comprise an important influence on natural gypsy moth populations.

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PYRROLIZIDINE ALKALOIDS IN AN OVERWINTERING POPULATION OF MONARCH BUTTERFLIES (*Danaus plexippus*) IN CALIFORNIA

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Abstract—California overwintering monarch butterflies contain both pyrrolizidine alkaloids (PAs) and their *N*-oxides. Analysis of 76 individual monarchs by TLC, HPLC, GLC, and GC-MS has shown the presence of three types of PAs, the saturated diester sarracine, the saturated monoester 7-angelylplatynecine, and the unsaturated dialcohol retronecine. Monarchs arriving at the overwintering site in Santa Cruz, California, showed a wide variation in both the type and amount of PA present. Those sampled after a PA-containing plant (*Senecio mikanioides*) had bloomed at the site showed an altered PA profile. While the plant was found to contain sarracine and 7-angelylplatynecine, which are nontoxic to mammals, the monarchs showed an increase in retronecine levels, a toxic PA, after the plant bloom. Apparently monarchs utilize PA-containing plants both en route to their overwintering site and at the site, and potentially alter those PAs to forms toxic to mammals.

Key Words—Monarch butterfly, *Danaus plexippus*, Lepidoptera, Danaidae, pyrrolizidine alkaloids, sarracine, 7-angelylplatynecine, retronecine, *Senecio mikanioides*, overwintering, defense.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) have been reported in over 300 plant species in six different families (Wrobel, 1985). Over 180 PAs have been identified to date, and the majority of these are mammalian hepatotoxins (McLean, 1970). Much attention has been focused recently on the use of PAs by a variety of

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insects as male pheromone precursors (Edgar, 1982; Edgar et al., 1971, 1973; Schneider et al., 1975, 1982; Conner et al., 1981; Komae et al., 1982; Krasnoff et al., 1987) and as chemical defense compounds (Brown, 1984; Boppré, 1986). All male danaine butterflies except the monarch, *Danaus plexippus*, appear to secrete PA-derived pheromones for courtship (Edgar, 1975; Ackery and Vane-Wright, 1984). The facts that monarch butterflies have the ability to store and accumulate PAs (Edgar et al., 1976) together with their known behaviors of imbibing PAs from withered and damaged plants (Pliske, 1975; Boppré, 1981) and by leaf-scratching (Boppré, 1983) indicate that monarchs gain some adaptive advantage in using PAs. The hypothesis has been advanced that monarchs utilize PAs as chemical defense compounds (Edgar, 1982; Edgar et al., 1976; Boppré, 1986; Kelley et al., 1987).

Kelley et al. (1987) recently showed that PAs are present in Mexican overwintering populations of monarchs, the first demonstration of PAs in North American monarchs. Monarchs in this population are believed to originate almost exclusively from the Great Plains to the Atlantic coast (Urquhart and Urquhart, 1976). This study examines the levels of PAs in a population of overwintering monarch butterflies collected in Santa Cruz, California, which is representative of coastal overwintering sites populated by monarchs from the western United States and, perhaps, western Canada.

METHODS AND MATERIALS

Sample Collection. Monarch butterflies and German ivy (*Senecio mikanioides*) florets were collected at Natural Bridges State Park, Santa Cruz, California, on December 22, 1986, and January 16, 1987. The December collection was prior to the plant bloom and the January collection was during the bloom. Florets (983 g) were collected in December, and 57 butterflies (33 males and 24 females) were captured in flight. Florets (949 g) were also collected in January, and 21 butterflies (13 males and 8 females) were collected nectaring on *Senecio mikanioides*. Individual butterflies and plant samples were placed in individual containers and frozen at −25°C until extracted.

Extraction of PAs. Fresh-frozen monarch butterflies were extracted individually as shown in Figure 1. Acidic methanol (10 ml) was added to a butterfly in a large test tube and the mixture was blended using a Polytron on medium speed for 1 min. After settling, the mixture was filtered under reduced pressure into a round bottom flask and the methanol was concentrated using a rotary evaporator. The extract was divided into two equal portions, and zinc dust was added to one of the portions. Both portions were then refrigerated overnight and treated identically as follows. Each portion was gravity filtered and the pH was adjusted to 10 with ammonium hydroxide. Volume was adjusted to 20 ml and

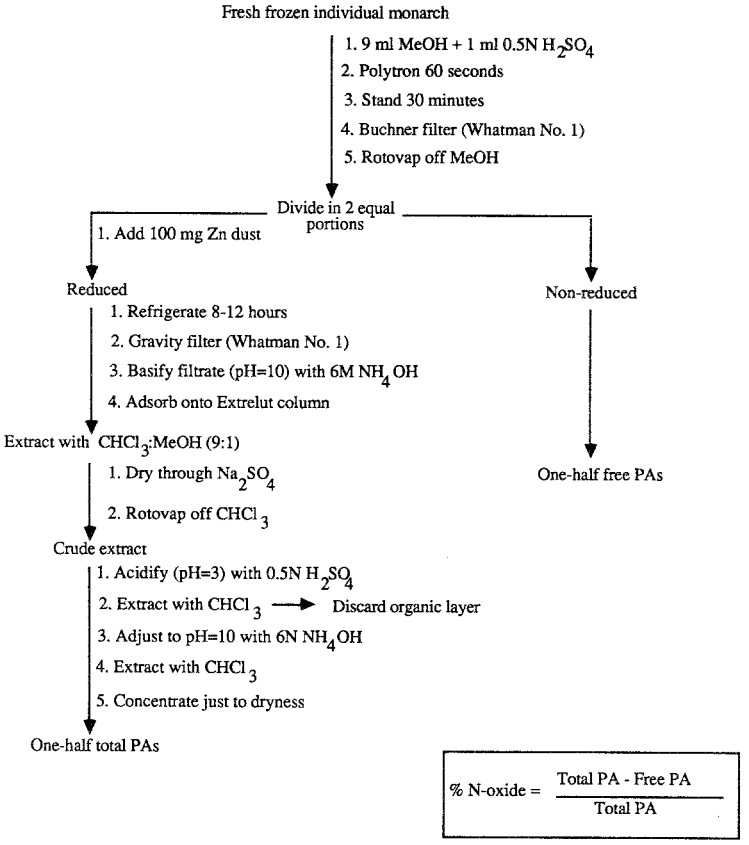

FIG. 1. Extraction scheme for pyrrolizidine alkaloids in monarch butterflies.

adsorbed onto an Extrelut QE column (20 ml capacity). These columns were used because of their selectivity for pyrrolizidine alkaloids (Brauchli et al., 1982). The column was eluted with 4 × 20-ml aliquots of 90% chloroform in methanol, dried through sodium sulfate, and the chloroform was rotary evaporated. The pH was then readjusted to 3 with sulfuric acid, and the concentrated eluate was extracted with three 10-ml aliquots of chloroform. This chloroform extract was discarded. The remaining aqueous portion was made basic to pH 10 with ammonium hydroxide and extracted three times with 10-ml aliquots of chloroform. This chloroform extract was rotary evaporated to dryness, transferred to tared vials with ethyl acetate, and concentrated for analysis.

The ground plant samples (ca. 1 kg) were first Soxhlet extracted with approximately 800 ml of methanol at 50°C for three days and then subjected

to the same extraction scheme except using larger volumes to accommodate the larger sample size. In an effort to maximize the total PAs extracted for subsequent identification, all plant extracts were reduced with zinc dust to convert all *N*-oxides to their free bases. Only total PA was analyzed from the plant. The free PA and reduced *N*-oxide fractions were examined separately for all butterfly extracts.

TLC Analysis. Plant samples were concentrated to 1.0 ml total volume and 10 μl were spotted on dried Silica gel 25 $\times$ 25-cm plates and developed twice with chloroform–methanol–ammonia 60:10:1 (CMA). Plates were dried, sprayed with *o*-chloranil to form pyrroles, heated, then sprayed with Ehrlich's reagent and reheated for 2 min (Molyneux and Roitman, 1980). Unsaturated PAs appeared as purple spots. Saturated PAs were visible as brownish purple spots only when present at high concentration because of their lack of pyrrole formation. A second solvent system of chloroform–methanol 75:25 (CM) was used with plates pretreated with lithium chloride (Huizing and Malingre, 1981) to provide an index of the relative polarities of PAs present in the plant.

A radial TLC system was used for separation and collection of individual PAs. A circular TLC plate (2-mm thickness) was mounted on a modified turntable and spun. Solvent was applied to the center of the spinning plate at a constant rate (9 ml/min) by a pump, and after the plate was saturated, the extract was applied in the same manner. The apparatus was illuminated by a UV light (254 nm) that allowed visualization of the bands of alkaloids as they moved towards the edge of the plate. Eluent was collected by a Golden Retriever fraction collector (5 ml/fraction). The solvent system was changed three times to provide better separation of the alkaloids. The first solvent system, 85 ml of a 95:4.5:0.5 chloroform–methanol–ammonia mixture, corresponded with the elution of the first band off the plate into the fraction collector. The second solvent system, a 90:10 chloroform–methanol mixture (100 ml), was used to rapidly elute the second, major UV-absorbing band on the plate. The third solvent, 100 ml of a 82.5:15.5:2 chloroform–methanol–ammonia mixture, eluted the remaining UV-absorbing bands on the plate. The plate was finally washed with 60 ml of ethanol.

There were 65 fractions collected in all; these were spotted on TLC silica gel plates, and fractions with the same TLC profile were combined for identification. Two major compounds were collected, one comprising fractions 15–31 and a second comprising fractions 35–50. The remaining fractions did not contain enough material of any one compound for NMR analysis, so were omitted from identification analysis. A total of 11.0 mg of material was collected in fractions 15–31 and 80.4 mg in fractions 35–50, both as gummy oils. The two major compounds were sufficiently pure for MS analysis, and the more abundant one was pure enough for NMR analysis.

HPLC Analysis. HPLC was used to separate and collect individual components from the plant extracts for identification. An alpha-cyclodextrin ana-

lytical column (25 cm × 4.6 mm ID, Advanced Separation Technologies, Inc.) was used with an Altex 110A pump and a water–acetonitrile 50:50 solvent system. Flow rate was 1.0 ml/min and UV detection was at 212 nm. A 50-μl sample loop was used for introducing the sample onto the column. Cyclodextrin columns are specifically designed for separation of nitrogen-containing optical isomers (Armstrong and Li, 1987) and performed adequately for separating most PAs present in the plant. Each run took approximately 1 hr.

GLC Analysis. All quantitation was performed using a Hewlett-Packard model 5890A gas chromatograph equipped with a 15-m DB-1 megabore column and an N/P detector. Carrier gas (helium) flow rate was 10 ml/min. Injector temperature was 230°C to minimize pyrolysis, and the oven temperature was programmed from 100°C to 150°C at 25°/min after an initial 2-min hold, then from 150°C to 250°C at 20°/min with a 10-min final hold. All PAs eluted within 10 mins. An HP 3390A integrator was used for recording and integrating the chromatograms. Monocrotaline was used as the external standard for quantitation, with the assumption made that all PAs responded to the N/P detector identically to monocrotaline.

Identification of PAs. Identification of individual PAs was done with a VG quadrupole mass spectrometer in chemical ionization mode (isobutane reagent gas) attached to an HP 5890A GC with a 30-m DB-5 column. Mass spectral data was combined with GLC retention time data and TLC retardation factors and compared with authentic standards. An NMR spectrum was obtained for sarracine, the most abundant PA, with a Varian EM390 NMR spectrometer (360 MHz). Deuterated chloroform was used as the solvent. The mass spectra and NMR spectrum for identified PAs agreed with spectra found in the literature (Roby and Stermitz, 1984). Sarracine: chemical ionization mass spectrometry (CIMS) *m/z* (rel. int.): M^+ 338 (50), 322 (63), 320 (42), 240 (25), 222 (41), 139 (86), 122 (100); [^{1}H]NMR ($CDCl_3$, 360 MHz): 6.34 (1H, q, J = 7.2 Hz, H-3′), 6.11 (1H, qq, J = 7.2, 1.4 Hz, H-3″), 5.29 (1H, br.t, H-7), 4.38 (1H, dd, J = 10.8, 7.0 Hz, H-9), 4.22 (1H, dd, J = 11.0, 7.0 Hz, H-9), 4.20 (2H, s, H-5′), 3.55 (1H, dd, J = 8.0, 3.7 Hz, H-8), 3.31 (1H, br.t, H-5), 3.17 (1H, m, H-3), 2.75 (1H, m, H-1), 2.73 (1H, m, H-3), 2.70 (1H, m, H-5), 1.96–2.08 (2H, m, H-2, H-6), 2.01 (3H, d, J = 7.4 Hz, H-4′), 1.99 (3H, dq, J = 7.4, 1.5 Hz, H-4″), 1.77–1.93 (2H, m, H-2, H-6), 1.88 (3H, dq, J = 1.6, 1.4 Hz, H-5″). 7-Angelylplatynecine: CIMS *m/z* (rel. int.) M^+ 240 (55), 205 (23), 140 (100), 122 (16), 109 (35).

RESULTS AND DISCUSSION

Sarracine (Figure 2) was the most abundant PA present in both the plant florets and the postbloom butterflies (Tables 1 and 2, Figure 3). The plant also contained 7-angelylplatynecine, retronecine, and 7-angelylretronecine in smaller

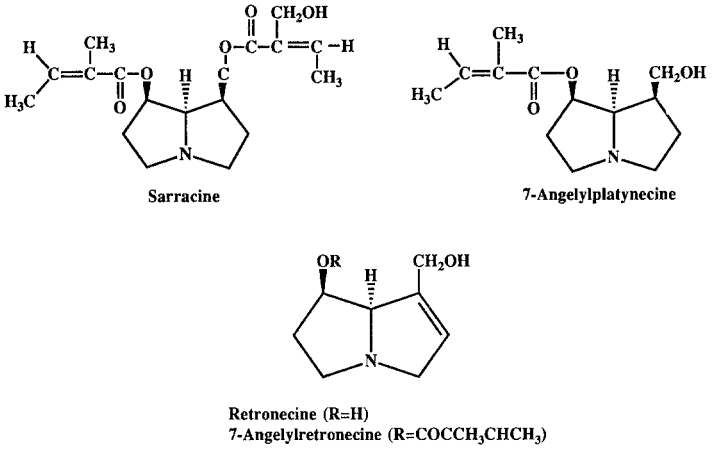

FIG. 2. Structures of pyrrolizidine alkaloids found in *Senecio mikanioides* and *Danaus plexippus* in the current study.

amounts (Table 1, Figures 2 and 3). The presence of these last three components in *Senecio mikanioides* has not been described before in the literature. Their identification was conducted by GC-MS and NMR. Only sarracine and its *N*-oxide were found in an earlier analysis (Culvenor and Geissman, 1961).

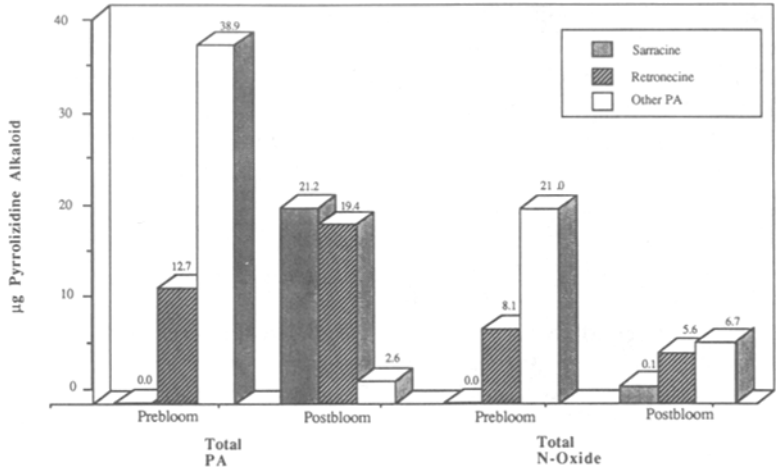

FIG. 3. PA content of monarch butterflies in Santa Cruz comparing saturated (sarracine) and unsaturated (retronecine) levels to the bloom of *Senecio mikanioides*. Values are mean amounts in µg per butterfly.

TABLE 1. PA CONTENT OF *Senecio mikanioides* FLORETS COLLECTED BEFORE AND AFTER FLOWERS BLOOMED[a]

	Prebloom	Postbloom
Total PA	1550	1240
Sarracine	1134 (73.2)	831 (67.0)
7-Angelylplatynecine	161 (10.4)	139 (11.2)
Retronecine	57 (3.7)	72 (5.8)
7-Angelylretronecine	25 (1.6)	68 (5.5)
Other PA (unidentified)	157 (10.1)	130 (10.5)

[a] Values are in μg PA per g wet plant tissue; numbers in parentheses are percent of total; 983 g used for prebloom sample, 949 g for postbloom sample.

A study on *Senecio vulgaris* (Hartmann and Zimmer, 1986) showed that the flower heads contained 66–79% of the total PA found in the plant, and increased from 0.32 μg/g wet tissue before blooming to 1.46 μg/g after blooming. Ninety percent of the PAs were in *N*-oxide form. The concentration of PAs in *Senecio mikanioides* was close to that found by Hartmann and Zimmer (Table 1). Hartmann and Zimmer did not Soxhlet extract the PAs in their study, but they stated that, while the percent of *N*-oxides decreased because of refluxing with methanol, the total PA present was unaffected.

Analysis of *Danaus plexippus* and *Senecio mikanioides* by TLC showed similar patterns (Table 3, Figure 4). Sarracine was the most mobile compound and had the highest R_f value for all solvent systems used. 7-Angelylplatynecine and retronecine were separated in all lithium pretreated plates, and 7-angelyl-

TABLE 2. PA PROFILE OF MONARCH BUTTERFLIES COLLECTED BEFORE AND AFTER BLOOM OF *Senecio mikanioides* IN SANTA CRUZ, CALIFORNIA

	Prebloom (N = 57)		Postbloom (N = 21)	
	Total PA	*N* oxide	Total PA	*N*-oxide
Total PA	51.6 ± 17.1[a]	29.1 ± 22.6	43.2 ± 6.7	14.2 ± 8.2
Sarracine	n.d.[b]	n.d.	21.2 ± 4.0	1.9 ± 1.2
7-Angelylplatynecine	n.d.	n.d.	n.d.	n.d.
Retronecine	12.7 ± 5.8	8.1 ± 4.6	19.4 ± 2.5	5.6 ± 2.8
7-Angelylretronecine	n.d.	n.d.	n.d.	n.d.
Other PA	38.9 ± 21.3	21.0 ± 19.7	2.6 ± 2.4	6.7 ± 4.7

[a] Values are μg PA per butterfly with standard deviations.
[b] n.d.—not detected (< 100 ng per butterfly).

TABLE 3. THIN-LAYER CHROMATOGRAPHY RETENTION FACTORS OF PYRROLIZIDINE ALKALOIDS FROM *Senecio mikanioides* FLORETS AND *Danaus plexippus*[a]

Compound	CMA system		CM system	
	R_f	R_{mono}	R_f	R_{mono}
Retronecine	0.03	0.09	0.28	0.72
7-Angelylretronecine	0.24	0.71	0.60	1.54
7-Angelylplatynecine	0.22	0.65	0.56	1.44
Sarracine	0.44	1.29	0.88	2.26

[a]Values are also given relative to monocrotaline, an external standard. See text for chromatographic conditions.

retronecine was distinguished from 7-angelylplatynecine by the positive response of the former to Ehrlich's reagent. The latter was visualized only with Dragendorff's reagent since its ring is saturated.

The major floret-derived alkaloids were isolated by preparative radial TLC.

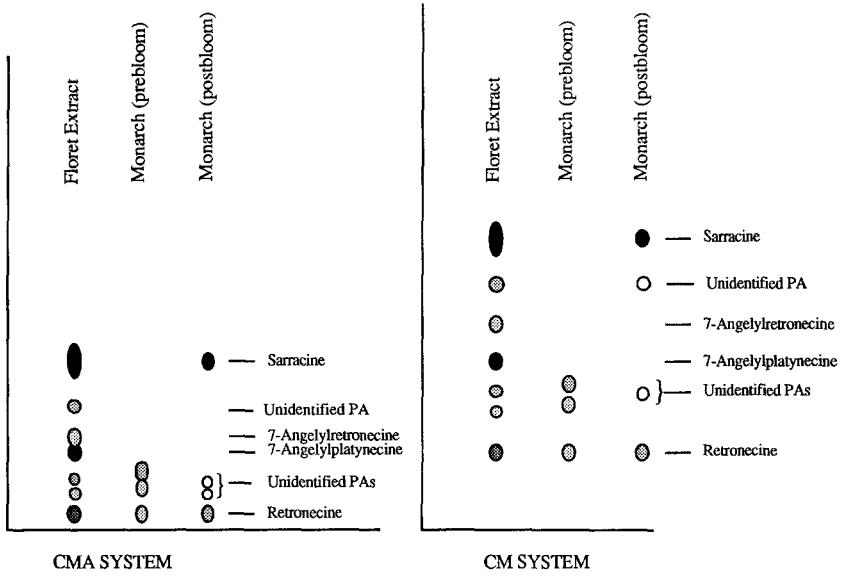

FIG. 4. Reconstructed thin-layer chromatogram plates illustrating typical pyrrolizidine alkaloid profiles from *Senecio mikanioides* florets and *Danaus plexippus* individuals. See text for conditions.

Fractions 15–31 were determined by GC-MS and NMR to be 7-angelylplatynecine with minor impurities. Fractions 35–50 were identified by GC-MS and NMR as sarracine (Roby and Stermitz, 1984).

Analysis of *Danaus plexippus* by GLC showed significant differences in PA content between prebloom and postbloom samples (Table 2). Males and females were combined in all analyses since no significant differences were seen between the sexes in either sample. The PA profile of the prebloom monarchs (Table 2) showed that the butterflies arrived in Santa Cruz with highly variable amounts of retronecine and other unidentified PAs. The total amount of PA per butterfly was not significantly different from that of the postbloom butterflies, but the relative amounts of individual PAs varied significantly (signed ranks test, $P < 0.01$). No monarchs examined before the bloom showed the presence of sarracine, demonstrating that the monarchs had no sources of this alkaloid en route to the overwintering site. This is supported by the fact that sarracine is an apparently rare alkaloid reported so far only in *Senecio mikanioides* and *Senecio atratus*, which is found only in the Colorado Rockies at 9,000–10,000 feet (Stermitz and Harris, 1987). *Senecio mikanioides* in this country has only been found at Natural Bridges State Park (Tuskes and Brower, 1978) and other scattered areas along the central California coast (Roitman, unpublished data).

A study by Kelley et al. (1987) on monarchs overwintering in Mexico showed higher total alkaloid content than the monarchs in this study (89 μg/butterfly versus 52 μg/butterfly), but their analysis used six individuals per sample, which prevented determination of individual variability. This population from Mexico contained both macrocyclic diester PAs (senecionine, integerrimine, and seneciphylline) and monoesters (echinatine, intermedine, and lycopsamine), perhaps derived from the nectar of species of Boraginaceae or Compositae imbibed during the southward fall migration. Thus, the population of California overwintering monarchs examined in the present study differed somewhat in quantities, and completely in PA structural types stored when contrasted with the sample from Mexico. The differences likely originate with the types of PA-containing plant species encountered by monarchs both in their migration to the sites and during nectar gathering at the sites. They found twice as much alkaloid in the females as in the males, which possibly could be due to transfer of PAs during mating earlier in the year (Boggs and Gilbert, 1979; Dussourd et al., 1988). No significant sex differences in amounts of PA present were found in the Santa Cruz population of monarchs.

Analysis of the PA profile of the postbloom monarchs showed that sarracine made up 49% of the total PA present. The proportion of PAs present as *N*-oxides declined from 56.3% prebloom to 34.7% postbloom. All of the butterflies contained sarracine, and this was the most abundant PA present in this sample. Surprisingly, sarracine *N*-oxide made up the smallest fraction of *N*-oxide in these monarchs, indicating either that little sarracine *N*-oxide was

present in the florets, that monarchs eliminated *N*-oxide after exposure to it, or that the majority was reduced to the free base in the monarch gut. The first possibility is doubtful based on the high percent of *N*-oxides shown to exist in *Senecio* florets during blooming (Hartmann and Zimmer, 1986) and from earlier work on *S. mikanioides* in Australia (Culvenor and Geissman, 1961), which showed that over 90% of the sarracine found was in its *N*-oxide form. The latter possibility is more likely since the free bases are more lipid soluble than the *N*-oxides, and reduction would increase the absorption of the alkaloid across the monarch gut and facilitate transport throughout the body. The reduction of keto to hydroxy cardenolides occurs efficiently in the gut of monarch larvae (Brower et al., 1982), but there are no data available for adult monarchs.

Retronecine levels increased by 53% (12.7 to 19.4 μg/individual) between pre- and post-bloom monarchs. Retronecine made up 45% of the total alkaloid in the postbloom monarchs, which is surprising since it comprises under 6% of the total alkaloid in the blooming plant. It is intriguing to examine this phenomenon in detail, and the possibilities are numerous. The monarchs might be selectively accumulating retronecine, metabolically converting other PAs such as 7-angelylretronecine to retronecine, or have exposure to another PA-containing plant near the study site that is enriched in retronecine. *Echium* sp. have been shown to contain both retronecine and heliotridine diesters such as echiumine and echimidine (Robins, 1982). A species of *Echium* is present near the park, but none of the typical PAs expected were found in the monarchs. This phenomenon awaits further examination.

The significance of the PAs found in the monarchs is yet to be determined. While cardenolides make the monarchs unpalatable to some birds such as blue jays (Brower et al., 1967), they do not make them unpalatable to other insects such as wasps, or to small mammals such as deermice. Both wasps and mice have been observed to feed on dead or dying monarchs at the California site, usually after a cold and windy or rainy night during which many monarchs are knocked off the eucalyptus trees onto the ground. Brown (1984) has shown that PAs can serve as predator deterrents against spiders, and Glendinning et al. (1988) have shown that monocrotaline can deter *Peromyscus* from feeding, but at levels much higher than those found in individuals in this study. It has also been suggested by Boppré (1986) that PAs are bitter and can serve as predator deterrents based solely on their taste characteristics. Even so, it is intriguing to speculate that the monarchs in Santa Cruz ingest putatively harmless saturated PAs such as sarracine and apparently concentrate levels of the PA retronecine, which is toxic to mammals for use in chemical defense—perhaps augmenting the cardenolide-based defense. The discovery that monarchs do not utilize dihydropyrrolizines as pheromones (Edgar, 1975; Edgar et al., 1971, 1973; Meinwald et al., 1968), or even need to use their hairpencils for successful mating (Pliske, 1975) lends support for the hypothesis that PAs might have some other function, such as in deterring predators. An alternative explanation

is that monarchs utilize *S. mikanioides* as a nectar source and only incidentally take in PAs; this does not appear to be as plausible based on the monarchs' ability to accumulate and store PAs (Edgar et al., 1976). Also, preliminary data on body part quantitation of PAs in these monarchs from this laboratory show that most is present in the exoskeleton, not in the abdomen as would be expected if the PAs were only incidentally imbibed.

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IDENTIFICATION OF A MINOR COMPONENT OF THE SEX PHEROMONE OF *Leucoptera malifoliella* (LEPIDOPTERA, LYONETIIDAE)

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Abstract—A new minor component in the female volatile extract of *Leucoptera malifoliella* (Costa) (Lepidoptera, Lyonetiidae) has been identified as 5,9-dimethyloctadecane (**2**). The amount detected of the minor compound **2** ranged from 4 to 8% in comparison with the major component 5,9-dimethylheptadecane (**1**). Neither compound has been found in the male volatile extract. The identification has been based on its spectroscopic properties and chromatographic behavior in comparison with an authentic synthetic sample. The synthesis has been carried out through a short route from 2,6-dimethylcyclohexanone (**3**). In field tests, compound **2** appears to act as a synergist of the major component **1** when mixed with the latter in a 100:0.1–5 ratio.

Key Words—Sex pheromone, *Leucoptera malifoliella*, identification, minor component, synergism, Lepidoptera, Lyonetiidae.

INTRODUCTION

The mountain-ash bentwing *Leucoptera malifoliella* (Costa) (Lepidoptera, Lyonetiidae), formerly *Leucoptera scitella* (Zeller), is considered one of the most important pests of orchards in many temperate regions of Europe and in the Central Asia mountains (Réal, 1966). Although the larvae are polyphagous, they feed preferentially on apple and pear trees, causing severe damage on the foliaceous tissue. The number of annual generations varies with the climate and

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season. In Italy four generations per year have been reported (Briolini, 1960; Ferro, 1961), one in England (Stainton, 1950), and two in other temperate areas of Europe. In the region of Catalunya, in northeast Spain, we have recorded three to four overlapping generations.

Because of the notable crop losses induced by this leafminer, monitoring and control of the pest by pheromones appears to be an attractive target. However and only until very recently, the major component of the female sex pheromone had not been identified. The compound, 5,9-dimethylheptadecane (**1**), is the first dimethyl-substituted hydrocarbon reported as a sex pheromone of a moth species (Francke et al., 1987). In other insects, branched hydrocarbons have also been sparsely found as sex pheromones. Thus, Roelofs and Cardé (1971) reported 2-methylheptadecane as sex pheromone and attractant of several species of Arctiidae, Sugie et al. (1984) identified 14-methy-1-octadecene in the peach leafminer moth *Lyonetia clerkella,* and Francke and coworkers (1988) found 5,9-dimethylpentadecane as sex pheromone of the coffee leafminer *Leucoptera coffeella.* Reported minor components are 2-methylhexadecane, 2-methyloctadecane, and 2-methylnonadecane in *Holomelina lamae* (Schal et al., 1987) and 5,9-dimethylhexadecane both in *Leucoptera scitella* and *Perileucoptera coffeella* (Francke et al., 1988). In Diptera, Carrière et al. (1988) have identified 3,7-dimethylnonadecane as the sex pheromone of the alfalfa blotch leafminer, *Agromyza frontella,* whereas several dimethyl- and trimethyl-substituted hydrocarbons of 37–40 carbon chain length have been reported as sex pheromones of tse-tse flies (Carlson et al., 1978).

In this paper, we describe the identification of 5,9-dimethyloctadecane (**2**) as a new minor component in the female volatile secretion of *Leucoptera malifoliella,* based on its spectroscopic properties and chromatographic behavior in comparison with an authentic sample. The compound has not been found in the male volatiles. In field tests, compound **2** significantly enhances the attractant activity of the major component **1** when mixed with the latter in a 100 : 0.1–5 ratio.

METHODS AND MATERIALS

Volatile Collection. Overwintering pupae were collected from infested orchards in several areas of Lérida province. The pupae were sexed with the aid of a 40× microscope and held over humidified cardboard pieces or apple tree leaves at 28°C and 18 : 6 hr light–dark cycle until emergence. For volatile collection, 1- to 2-day-old unmated males and females were kept for 2–3 days in a 1-liter Erlenmeyer flask, through which a purified airstream was passed at a flow rate of ca. 1 ml/min. The air was purified on a Porapak Q 60–80 mesh

filter, which had been previously conditioned as already described (Cross et al., 1976). The emitted volatiles were condensed on a U-tube cooled to −78°C, which was rinsed twice a day with nanograde hexane. The extract was concentrated to a 200-μl volume under a gentle stream of nitrogen for identification.

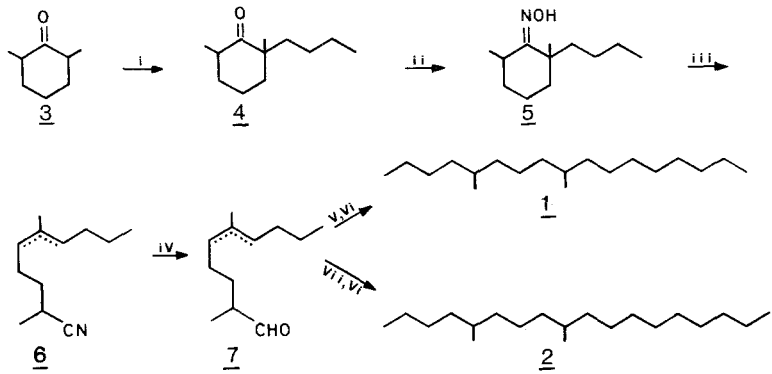
Identification. Gas chromatographic analyses were carried out on Carlo Erba Mega 5160 and 4130 models, equipped with a split–splitless dual-mode injection system, provided with a FID detector. Three fused silica capillary columns of different polarities were used: SPB-1, 25 m × 0.25 μm ID (column A); SPB-35, 15 m × 0.25 μm ID (column B); and Supelcowax-10, 30 m × 0.25 μm ID (column C). Gas chromatographic conditions were as follows: injector temperature: 250°C, detector temperature: 250°C, column temperature: isothermal at 100°C for 6 min, then programmed to 250°C at 4°C/min and held at this temperature for 15 min. Hydrogen (0.5 ml/min) was used as carrier gas.

GC-MS analyses were conducted on a HP-5995 at 70 eV in the EI mode, whereas a HP-5988A model with methane as ionizing gas was utilized in the CI mode. The high-resolution NMR spectrum of ca. 20 μg of the active material was recorded on a Varian XL-200 (200 MHz), using C_6D_6 as solvent in a 60-μl spherical microcell.

Synthesis. Boiling points were determined on a Kugelrohr distillation apparatus and are uncorrected. IR spectra were recorded in CCl_4 solution on a Perkin Elmer 399B grating spectrometer. [^{1}H]NMR spectra were determined in $CDCl_3$ solution on a Bruker WP80SY spectrometer operating at 80 MHz, and absorptions are expressed in δ scale relative to TMS. GLC analyses were performed on a Carlo Erba Vega 6000 model, equipped with a FID detector, using a 3% OV-101 glass column, 2 m × 3 mm ID, on Chromosorb W (nitrogen as carrier gas).

Reactions requiring anhydrous and oxygen-free conditions were performed under a dried inert atmosphere (N_2). Anhydrous solvents were prepared as follows: tetrahydrofuran (THF) by distillation from Na/benzophenone, hexane from Na, dimethylformamide (DMF) from CaH_2 and pyridine from KOH.

*2-n-Butyl-2,6-dimethylcyclohexanone (**4**).* This compound was prepared by a modification of the Kocienski procedure (1977) (Scheme 1). Thus, in a 250-ml, three-neck, round-bottom flask with a magnetic stirrer, reflux condenser, addition funnel, and nitrogen inlet was placed 2.16 g (45 mmol) of a 55% oil dispersion of NaH. After removing the mineral oil with pentane, 45 ml of anh. THF and 5 ml of anh. DMF were added. The mixture was heated to reflux and 4.72 g (37.5 mmol) of 2,6-dimethylcyclohexanone (**3**) was added slowly. When gas evolution had subsided, the mixture was cooled to room temperature and 5.0 g (37.5 mmol) of *n*-butyl bromide added. The reaction mixture was stirred for 1 hr at room temperature and then refluxed for 1 hr more. Then 25 ml of



i: NaH, n—C_4H_9Br/THF-DMF (30%); ii: $NH_2OH.HCl$, AcONa/EtOH (92%); iii: TsCl/pyridine (64%); iv: DIBAH/hexane (83%); v: Ph_3P^+n-$C_7H_{15}Br^-$, n-BuLi/THF (60%); vi: H_2, Pd/C (86%); vii: Ph_3P^+n—$C_8H_{17}Br^-$, n—BuLi/THF (67%).

SCHEME 1. Synthetic route to compounds **1** and **2**.

2N H_2SO_4 was added, and the reflux continued for 2 hr. After cooling to room temperature, the organic layer was decanted, and the aqueous phase was extracted with ether (3× 30 ml), washed with brine, and dried ($MgSO_4$). The solvent was stripped off, and the residue was fractionally distilled under reduced pressure to afford a mixture of 1.34 g (28%) of the starting ketone **3**, which could be recycled, and 2.06 g (42% from unreacted starting material) of the desired ketone **4**, bp 90–95°C/12 torr.

IR: ν 2920, 1700, 1455, 1375 cm^{-1}. [^{1}H]NMR: δ 0.87 (t, 3H, J = 6.5 Hz, $CH_2C\underline{H}_3$), 0.97 (s, 3H, $C\underline{H}_3CCH_2$), 0.95 (d, 3H, J = 7.5 Hz, $C\underline{H}_3CH$), 1.05–2.1 (c, 12H, $6CH_2$), 2.55 (m, 1H, CHCO). MS m/z (relative intensity): 182 (M^+, 18), 139 (7), 127 (60), 126 (100), 124 (76), 112 (11), 111 (71), 109 (30), 98 (18), 97 (45), 96 (10), 95 (61), 84 (26), 83 (24), 82 (36), 81 (15), 70 (25), 69 (56), 68 (13), 67 (14), 57 (18), 56 (51), 55 (75), 53 (11), 43 (16), 41 (43).

2-n-Butyl-2,6-dimethylcyclohexanone Oxime (5). A mixture of 1.6 g (8.8 mmol) of ketone **4**, 3.37 g (48.5 mmol) of hydroxylamine hydrochloride, 6.7 g (48.5 mmol) of sodium acetate trihydrate, and 15 ml of ethanol was refluxed for two days. After quenching with water and extracting with ether, the organic layer was washed with brine (4× 20 ml), dried ($MgSO_4$), and concentrated under vacuum. The residue was distilled to give 1.60 g (92%) of oxime **5** as a colorless oil, bp 127–135°C/10 torr.

IR: ν 3230, 2920, 1455, 1375, 1285 cm^{-1}. [^{1}H]NMR: δ 0.90 (t, 3H, J = 6.5 Hz, $CH_2C\underline{H}_3$), 1.10 (s, 3H, CH_3C isomer Z), 1.18 (s, 3H, CH_3C isomer E), 1.16 (d, 3H, J = 7.5 Hz, $C\underline{H}_3CH$ isomer Z), 1.21 (d, 3H, J = 7.5 Hz, $C\underline{H}_3CH$ isomer E), 1.1–2.0 (c, 13H, $6CH_2$ and CH). MS m/z (relative inten-

sity): 197 (M^+, 5), 180 (10), 154 (20), 142 (17), 141 (100), 126 (17), 124 (17), 113 (10), 109 (11).

Beckmann Fragmentation to Olefinic Nitriles ***6***. The procedure described by Marshall et al. (1970) was used. Thus, a mixture of 1.5 g (7.6 mmol) of oxime ***5*** and 3.3 g (17 mmol) of *p*-toluensulfonyl chloride in 5 ml of anh. pyridine was heated to reflux for 3 hr, cooled to room temperature, and quenched by pouring over 50 ml of ice water. After extraction with hexane (3× 50 ml), the combined organic layers were washed with brine and dried ($MgSO_4$). The solvent was removed under vacuum to furnish a brown oil, which was distilled to yield 0.87 g (64%) of the isomeric olefinic nitriles **6**, bp 90–95°C/0.1 torr. In addition to the major isomers obtained, which contained the expected trisubstituted double bonds on both sides of the methyl group at C-6, the isopropylidene derivative (δ 4.71) was also detected as minor compound.

IR: ν 2920, 2230, 1450, 1380 cm^{-1}. [^{1}H]NMR: δ 0.85 (t, 3H, J = 7 Hz, $CH_2C\underline{H}_3$), 1.25 (d, 3H, J = 7.7 Hz, $C\underline{H}_3CH$), 1.2–1.8 (c, 9H, $3CH_2CC$ and $CH_3C{=}C$), 1.8–2.3 (m, 4H, $2CH_2C{=}C$), 2.4 (m, 1H, CHCN), 5.05 (t, 1H, J = 6.0 Hz, CH=C). MS (major isomer) *m/z* (relative intensity): 179 (M^+, 7), 164 (21), 151 (57), 150 (35), 136 (46), 122 (28), 109 (20), 108 (100), 97 (12), 95 (11), 94 (18), 69 (15), 67 (11), 55 (18), 41 (14).

Reduction of Nitriles ***6*** *to Aldehydes* ***7***. To a magnetically stirred solution of 0.54 g (3 mmol) of mixture of nitriles **6** in 10 ml of anh. hexane was added, dropwise at −78°C, 3.35 ml of a 20% DIBAH solution in hexane (3.3 mmol). The solution was stirred at this temperature for an additional 30 min., warmed to room temperature, and further stirred for 2 hr. The mixture was poured carefully over 35 ml of a 3 M H_2SO_4 solution with stirring. The organic layer was decanted, and the aqueous phase was extracted with hexane (3× 40 ml). The organic extracts were combined and washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was distilled in a bulb-to-bulb distillation apparatus to give 0.45 g (83%) of mixture of aldehydes **7**, bp 100–104°C/0.1 torr.

IR: ν 2920, 1725 cm^{-1}. [^{1}H]NMR δ 0.85 (t, 3H, J = 5.5 Hz, $CH_2C\underline{H}_3$), 1.1 (d, 3H, J = 7.2 Hz, $C\underline{H}_3CH$), 1.2–1.8 (c, 9H, $3CH_2CC$ and $CH_3C{=}C$), 1.8–2.1 (m, 4H, $2CH_2C{=}C$), 2.3 (m, 1H, $C\underline{H}CHO$), 5.08 (t, 1H, J = 6.0 Hz, CH=C), 9.60 (d, 1H, J = 2.4 Hz, CHO). MS (major isomer) *m/z* (relative intensity): 182 (M^+, 8), 164 (28), 135 (15), 126 (50), 125 (24), 124 (100), 109 (59), 97 (20), 95 (100), 83 (20), 82 (28), 81 (37), 69 (38), 67 (28), 55 (53), 41 (33).

5,9-Dimethylheptadecane ***(1)***. In a flame-dried, three-neck, 50-ml, round-bottom flask equipped with magnetic stirrer, nitrogen inlet, and gas bubbler was placed 1.6 g (3.6 mmol) of *n*-heptyltriphenylphosphonium bromide in 15 ml of anh. THF. The mixture was cooled to −78°C and 3.3 ml of 1 M *n*-BuLi in

hexane (3.3 mmol) was added. After stirring at this temperature for 30 min, the hydrogen evolution had ceased. Then 0.45 g (2.47 mmol) of a mixture of aldehydes **7** in 2 ml of anh. THF was added and the reaction mixture stirred for an additional 30 min at −78°C and 1 hr at room temperature. After quenching with 25 ml of a 1 M H_2SO_4 soln., the organic layer was separated and the aqueous phase extracted with hexane (3× 20 ml). The organic phases were combined, washed with brine, and dried ($MgSO_4$) to leave a residue, after evaporation of the solvent, that was purified by column chromatography on silica gel eluting with hexane. The resulting oil was distilled in a bulb-to-bulb distillation apparatus to afford 0.40 g (60%) of mixture of the corresponding diunsaturated olefins, bp 119–123°C/0.1 torr.

IR: ν 3020, 2995, 2920, 1450, 1375 cm^{-1}. [^{1}H]NMR δ 0.90 (t, 6H, J = 6.5 Hz, $2CH_2C\underline{H}_3$), 0.95 (d, 3H, J = 6.5 Hz, $C\underline{H}_3CH$), 1.1–1.5 (b, 14H, $7CH_2CC$), 1.5–1.75 (dm, 3H, $CH_3C{=}C$), 1.8–2.2 (m, 6H, $3CH_2C{=}C$), 2.45 (m, 1H, CHC=C), 4.95–5.5 (m, 3H, 3CH=C). MS (major isomer) m/z (relative intensity): 264 (M^+, 15), 207 (29), 180 (22), 179 (49), 166 (20), 165 (32), 151 (25), 124 (44), 123 (37), 111 (23), 110 (38), 109 (71), 97 (22), 96 (37), 95 (100), 83 (29), 89 (23), 81 (67), 69 (52), 68 (23), 67 (22), 55 (41), 41 (20).

A mixture of 0.3 g (1.13 mmol) of the olefins, 30 mg of 10% Pd/C, 3 ml of THF, and 8 ml of methanol was hydrogenated at atmospheric pressure and room temperature. When the required amount of hydrogen had been absorbed, the catalyst was removed by filtration and the solvent evaporated off under vacuum. The resulting residue was purified by column chromatography on silica gel eluting with hexane and distilled in a bulb-to-bulb distillation apparatus to yield 0.26 g (86%) of hydrocarbon **1** (>98% on GLC analysis).

IR: ν 2920, 1460, 1375 cm^{-1}. [^{1}H]HMR δ 0.83 (d, 6H, J = 6.4 Hz, 2 $C\underline{H}_3CH$), 0.90 (t, 6H, J = 6.4 Hz, $2C\underline{H}_3CH_2$), 1.0–1.7 (b, 28H, $13CH_2$, and 2CH). MS m/z (relative intensity): 268 (M^+, 12), 253 (12), 239 (7), 211 (50), 210 (12), 183 (16), 155 (56), 154 (25), 141 (20), 140 (43), 127 (12), 113 (12), 112 (11), 99 (16), 97 (11), 85 (64), 84 (24), 71 (48), 70 (13), 69 (15), 57 (87), 56 (21), 55 (30), 43 (100), 41 (56).

5,9-Dimethyloctadecane (2). Following the same procedure described for the synthesis of **1**, starting from 1.30 g (2.86 mmol) of *n*-octyltriphenylphosphonium bromide, 2.6 ml of 1 M *n*-BuLi (2.6 mmol) in hexane, and 0.4 g (2.2 mmol) of aldehydes **7**, the corresponding mixture of intermediate olefins (0.41g, 67%) was obtained after purification on silica gel.

IR: ν 3020, 2995, 2920, 1450, 1375 cm^{-1}. [^{1}H]NMR δ 0.90 (t, 6H, J = 6.5 Hz, $2C\underline{H}_3CH_2$), 0.95 (d, 3H, J = 6.5 Hz, $C\underline{H}_3CH$), 1.1–1.5 (b, 16H, $8CH_2CC$), 1.5–1.8 (dm, 3H, $CH_3C{=}C$), 1.8–2.2 (m, 6H, $3CH_2C{=}C$), 2.45 (m, 1H, CHC=C), 5.0–5.5 (m, 3H, 3CH=C). MS (major isomer) m/z (relative intensity): 278 (M^+, 15), 221 (18), 194 (22), 193 (38), 165 (36), 151 (29),

124 (59), 123 (39), 111 (25), 110 (59), 109 (72), 97 (21), 96 (26), 95 (100), 83 (23), 82 (29), 81 (74), 69 (55), 68 (24), 67 (24), 55 (48), 41 (25).

Hydrogenation of 0.15 g (0.5 mmol) of a mixture of olefins, in the presence of 15 mg of 10% Pd/C in 5 ml of THF and 10 ml of methanol, afforded, under the above reaction conditions, 0.13 g (86%) of compound **2** after purification on silica gel (>98% purity on GLC analysis), bp 125–126°C/0.1 torr.

IR: ν 2920, 1460, 1375 cm^{-1}. [^{1}H]NMR δ 0.81 (d, 6H, J = 6.4 Hz, 2C$\underline{H_3}$CH), 0.90 (t, 6H, J = 6.4 Hz, 2C$\underline{H_3}CH_2$), 1.0–1.7 (b, 30H, 14CH_2, and 2CH). MS m/z (relative intensity): 282 (M^+, 9), 267 (11), 253 (5), 225 (44), 224 (13), 197 (12), 196 (8), 155 (58), 154 (57), 127 (9), 126 (12), 113 (12), 112 (9), 99 (15), 85 (57), 84 (24), 71 (44), 70 (11), 69 (13), 57 (88), 56 (21), 55 (31), 43 (100), 41 (43).

Field Tests. The field trials were carried out in infested apple orchards near the villages of Roselló (Lérida) and Mas Badía (Gerona) (June–September 1988–1989). Delta traps containing the lures were hung on trees at a height of 1.8 m and 25–30 m apart. Polyethylene vials (3 cm high × 1.1 cm ID) containing mixtures of 1 mg of compound **1** and minor amounts of **2** were used as dispensers. The traps were arranged in randomized blocks and rotated twice a week. Three replicates of each formulation were tested at each site. Trap catches were subjected to $\sqrt{x+1}$ transformation followed by analysis of variance, and the data were analyzed statistically for significance according to LSD and Tuckey's HSD test.

RESULTS AND DISCUSSION

In preliminary experiments carried out in 1986–1987, 450 1- to 2-day-old virgin females were macerated in nanograde methylene chloride for 1 hr. After filtration, the extract was carefully concentrated under a gentle stream of nitrogen and subjected to a flash distillation under high vacuum (0.01 torr) to remove any contaminating lipidic material. The volatile fraction, which turned out to be as active as the crude extract, was fractionated on silica gel eluting with mixtures of hexane–ether according to the procedure of Buser and Arn (1975). Among the fractions tested, only those eluted with hexane and hexane–ether 5% were significantly active in field trials. On the other hand, 150 female-equivalents were subjected to a series of microchemical reactions such as hydrogenation (H_2, Pd/C), saponification (KOH/MeOH), acetylation (Ac_2O/py), and reduction (LAH/ether) with no appreciable loss of activity. The [^{1}H]NMR spectrum of ca. 20 μg of the most active fraction in C_6D_6 showed, as noticeable absorptions, a doublet at δ 0.88 (J = 7.2 Hz), a distorted triplet at δ 0.93 (J = 7.0 Hz), and a broad complex absorption centered at δ 1.29. These results point out the presence of saturated hydrocarbon(s) in the active extract as described previously (Francke et al., 1987).

In order to look for possible minor components that might enhance the male attractant activity of the major compound **1**, a careful analysis of the active material was undertaken. Gas chromatographic analysis of the volatiles released by 250 virgin calling females, on three different fused silica capillary columns (A, B, and C, see above), showed the presence of two compounds in 92:8 to 96:4 ratio. The retention times of both compounds were the following: 30.75 and 33.85 min in column A, 25.48 and 29.03 min in column B, and 26.40 and 30.28 min in column C. It must be noted that neither compound **1** nor **2** was found in the volatile extract of 102 unmated males under the chromatographic conditions specified above (Figure 1).

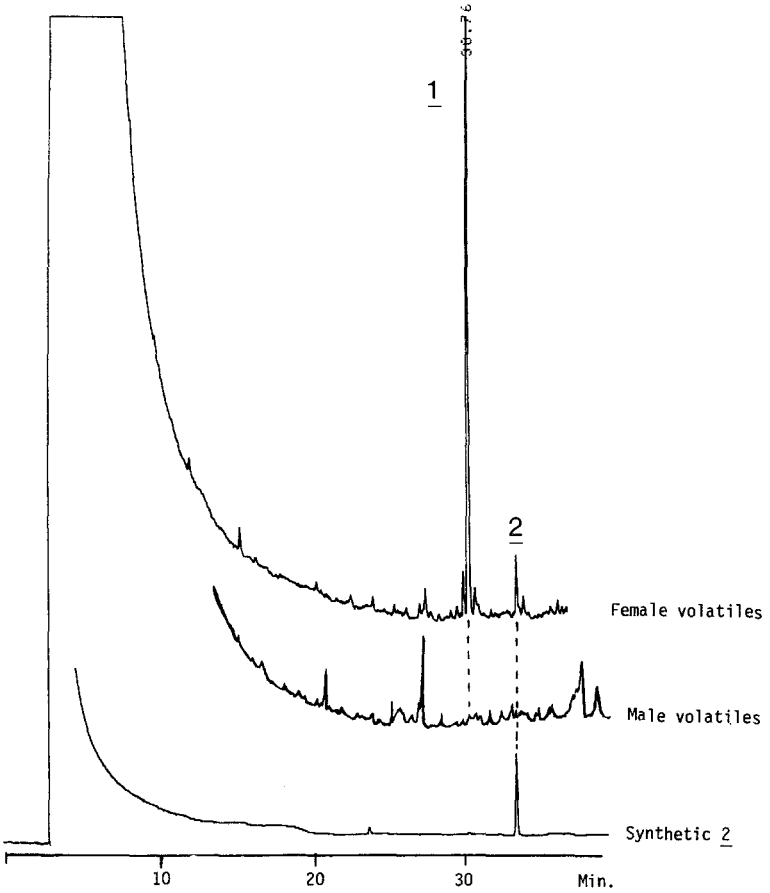


FIG. 1. (Upper) GC trace of the female volatile extract of *Leucoptera malifoliella* using column A (see text). (Middle) GC trace of the male volatile extract of *Leucoptera malifoliella* under the same chromatographic conditions. (Lower) GC trace of the synthetic minor component **2**.

The EI mass spectrum of the major compound showed the highest mass ion at *m/z* 268 and diagnostic peaks at *m/z* 253, 211, and 155, suggesting a 5,9-dimethyl branched saturated hydrocarbon (Pomonis et al., 1980). On the other hand, the CI/CH_4 mass spectrum showed the base peak at *m/z* 267, corresponding to M^+-1, in agreement with the previously assigned molecular weight. Finally, comparison of the chromatographic retention time on columns A, B, and C with a synthetic sample confirmed the structure of the major component as 5,9-dimethylheptadecane (**1**), as already described (Francke et al., 1987). Compound **1** has been utilized by us to monitor the population density of the pest for the field trials, as well as to establish three to four annual overlapping generations in Lérida province.

On the other hand, the EI mass spectrum of the minor compound showed the highest mass peak at *m/z* 282 along with other prominent, characteristic peaks at *m/z* 267, 225, and 155. The molecular weight of 282, corresponding to a $C_{20}H_{42}$ structure, was confirmed by the CI/CH_4 mass spectrum, which showed the base peak at *m/z* 281 (M^+-1). In addition, the peak at *m/z* 267, which suggested the favored loss of a methyl group from the molecular ion, supported a methyl-branched hydrocarbon structure. On the other hand, the ion of *m/z* 225 pointed to a $C_{16}H_{33}$ fragment, which implied a branch point at C-5, whereas that of *m/z* 155 was assigned to a $C_{11}H_{23}$ fragment resulting from the α cleavage on both sides of a ramification at C-9. This caused an enhancement of the peak in comparison with the same ion in the mass spectrum of **1**, wherein only one favored fragmentation with that mass is possible. Therefore, after considering the fragmentation patterns of other dimethyl-branched hydrocarbons (Pomonis et al., 1980), we finally assigned the structure of the minor compound as 5,9-dimethyloctadecane (**2**). In addition, a plausible biogenetic consideration is that the possible precursor of both compounds **1** and **2** might be a C-18 acid structure, whose carbonyl group would be lost to produce **1** or transformed into a methyl group by a reduction–elimination process to give rise to **2** (Francke, personal communication).

The assignment of the minor component **2** was confirmed by comparison of the chromatographic behavior (retention time) on columns A, B, and C (see above) and the EI and CI mass spectra with those of an authentic sample of **2** prepared by synthesis (Figures 1 and 2). The synthetic route to compounds **1** and **2** followed a modification of the procedure described by Kocienski and Ansell (1977) (Scheme 1). Thus, base-catalyzed alkylation of 2,6-dimethylcyclohexanone (**3**) with *n*-butyl bromide afforded ketone **4**, which, after transformation into the oxime **5**, was subjected to a Beckmann fragmentation according to the procedure described by Marshall et al. (1970). Treatment of the oxime **5** with *p*-toluensulfonyl chloride in pyridine at reflux furnished a mixture of nitriles **6** in good yield, with no trace of the corresponding lactam being detected. As shown by the [^{1}H]NMR spectrum, the mixture contained mainly the isomeric nitriles with the expected trisubstituted double bonds on both sides of the methyl

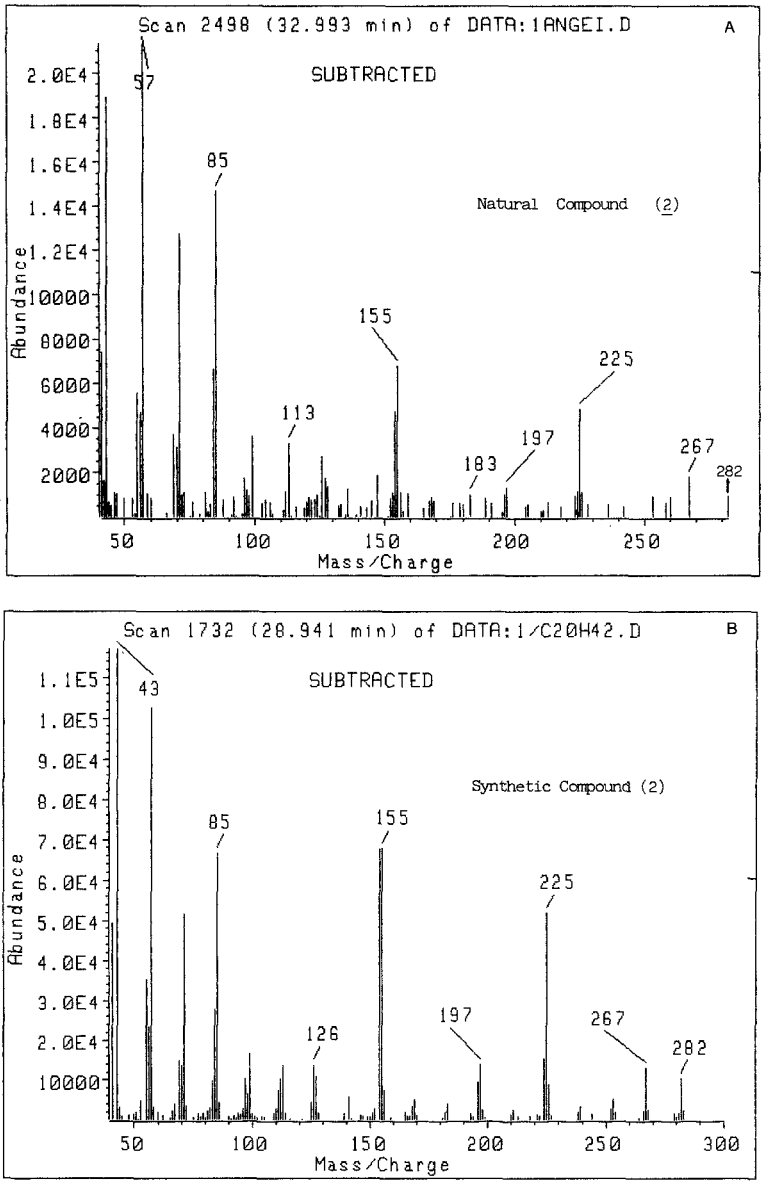


FIG. 2. (A) EI-MS of the natural minor component **2**. (B) EI-MS of the synthetic compound **2**.

group at C-5, although the presence of the corresponding isopropylidene isomer was also noted (δ 4.71) in minor amount (ca. 12%). Reduction of nitriles **6** with DIBAH in hexane at −78°C furnished a mixture of aldehydes **7**, which were transformed into compounds **1** and **2** by Wittig reaction with the required ylides followed by complete hydrogenation with Pd/C. The overall yield of both routes was ca. 12% from ketone **3**.

In preliminary field tests carried out in 1988, the minor compound **2** appeared to enhance the activity of the major component **1**. Thus, when compound **2** was mixed with **1** at 0.1–1% level, a significant increase of catches was observed in comparison with captures of compound **1** alone (Table 1). The synergistic effect was confirmed in new field experiments, run in 1989, wherein baits loaded with mixtures of compounds **1** and **2** in 100:1 to 100:5 ratios showed higher attractant activity than compound **1** alone (Table 2). However,

TABLE 1. CAPTURE OF *Leucoptera malifoliella* MALES WITH BLENDS OF COMPOUNDS **1** AND **2** (ROSELLÓ, LÉRIDA, JUNE–SEPTEMBER 1988)

Composition of lure (mg)			
1	**2**	Ratio **2:1**	Mean catch/trap/week[a]
1		0:100	60.6 b
1	0.001	0.1:100	117.2 a
1	0.01	1:100	109.2 a
	1	100:0	1.8 c

[a] Three replicates. Means followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's HSD test.

TABLE 2. CAPTURE OF *Leucoptera malifoliella* MALES WITH MIXTURES OF COMPOUNDS **1** AND **2** (ROSELLÓ, LÉRIDA, JUNE–JULY 1989)

Composition of lure (mg)			
1	**2**	Ratio **2:1**	Mean catch/trap/week[a]
1		0:100	67.7 c
1	0.001	0.1:100	77.3 bc
1	0.01	1:100	84.0 ab
1	0.05	5:100	105.0 a
	1	100:0	3.5 d

[a] Three replicates. Means followed by the same letter are not significantly different at $P = 0.05$ according to LSD test.

TABLE 3. CAPTURE OF *Leucoptera malifoliella* MALES WITH BLENDS OF COMPOUNDS **1** AND **2** (MAS BADÍA, GERONA, JUNE–JULY 1989)

Composition of lure (mg)			
1	**2**	Ratio **2:1**	Mean catch/trap/week[a]
1		0:100	34.1 b
1	0.001	0.1:100	35.2 ab
1	0.01	1:100	36.8 ab
1	0.05	5:100	45.2 a
	1	100:0	3.4 c

[a]Three replicates. Means followed by the same letter are not significantly different at $P = 0.05$ according to LSD test.

in orchards with a lower level of infestation (Mas Badía, 1989), the synergism was noted only in lures containing 5% of the minor component **2** (Table 3), clearly the best formulation found in the former field test. In all cases, the intrinsic attractant activity of **2** was very low.

In summary, a new minor component **2** has been found in the female volatile extract of *Leucoptera malifoliella*. The compound has been identified as 5,9-dimethyloctadecane based on its spectroscopic properties and chromatographic behavior in comparison with an authentic synthetic sample. The structure of the major component as 5,9-dimethylheptadecane also has been confirmed after comparison with an independently prepared **1**. The synthesis of both compounds has been carried out through a short route from 2,6-dimethylcyclohexanone. In field tests, the minor component **2** appears to act as a synergist of the major compound **1** when mixed with the latter in a 100:0.1–5 ratio.

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DIFFERENCES IN OXIDASE AND ESTERASE ACTIVITIES INVOLVED IN PHEROMONE BIOSYNTHESIS IN TWO SPECIES OF *Choristoneura*

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Abstract—The released pheromone and the glandular lipids, labeled with [^{14}C]acetate, were analyzed from *Choristoneura orae* and *Choristoneura fumiferana* budworm moths by thin-layer chromatography and autoradiography. Radiolabeled lipids in the gland appeared to be identical in the two moths with both insects containing high amounts of 11-tetradecenyl acetates. In contrast, the *C. orae* moths released primarily labeled acetate ester and alcohol, and the spruce budworm moths (*C. fumiferana*) labeled aldehyde consistent with the known composition of their respective pheromones. The levels of the enzymes responsible for converting the acetate ester into aldehyde were found to be significantly lower in gland extracts from *C. orae* moths than from *C. fumiferana* moths. These results implicate an acetate esterase and an alcohol oxidase in controlling the composition of the pheromone blend released from closely related budworm species.

Key Words—Acetate ester, acetate esterase, alcohol, oxidase, *Choristoneura fumiferana*, *Choristoneura orae*, Lepidoptera, Tortricidae, pheromone, spruce budworm, biosynthesis.

INTRODUCTION

The primary components of the sex pheromone of the eastern spruce budworm [*Choristoneura fumiferana* (Clem.)] are a blend of *E*-11- and *Z*-11-tetradecenal in a molar ratio of 96:4 (Weatherston et al., 1971; Sanders and Weatherston, 1976; Silk et al., 1980). In the pheromone-producing gland, the Δ11-tetradecenyl acetate esters serve as storage precursors and are converted into the pher-

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omone in reactions catalyzed by enzymes with acetate esterase and alcohol oxidase activities. This conclusion is supported by the high levels of acetate ester in the gland (Silk et al., 1980), the decrease in the amount of labeled ester on release of the pheromone, and identification of the acetate esterase and alcohol oxidase in gland extracts (Morse and Meighen, 1984a,b). The alcohol oxidase and a developmentally regulated fatty alcohol acetyltransferase catalyzing the synthesis of the acetate ester precursor appear to be specifically located in the gland (Morse and Meighen, 1987a,b). It has been proposed that pheromone differences in functional groups (ester, alcohol, aldehyde) in closely related species are controlled by the relative levels of acetate esterase and alcohol oxidase activities (Morse and Meighen, 1986).

Choristoneura orae (Free.) budworms are closely related to the spruce budworm. Early studies of field catches of *C. orae* indicated that the pheromone is composed primarily of tetradecenyl acetates (Sanders, 1971; Sanders et al., 1974). More recent studies have demonstrated that the *C. orae* pheromone consists of *E*-11- and *Z*-11-tetradecenyl acetate esters and tetradecenols in a 90 : 10 ratio with a *E*/*Z* ratio (8 : 1) similar to that found in the *C. fumiferana* aldehyde pheromone (Gray et al., 1984). In this report, the lipids and enzymes in gland extracts and the released pheromone have been analyzed from *C. orae* budworm moths and compared to the analogous data for spruce budworm moths. The results provide evidence that the acetate esterase and alcohol oxidase enzymes are directly responsible for generating the different pheromone blends in the two budworm moths.

METHODS AND MATERIALS

Materials. All long-chain compounds (>12 carbons), $\geq 95\%$ pure as judged by gas chromatography, were obtained from ChemSampCo. All other substrates were obtained from Sigma. [1-^{14}C]Acetate (56 mCi/mmol) was obtained from Amersham. [^{3}H]Acetyl-CoA (200 mCi/mmol) was synthesized according to the method of Stadtman (1957) from [^{3}H]acetic anhydride (400 mCi/mmol, New England Nuclear) and coenzyme A. Phosphate buffers were prepared by mixing appropriate amounts of K_2HPO_4 and NaH_2PO_4. All solvents were reagent grade and supplied by Fisher.

In Vivo Labeling. Female budworm moths were received as pupae and allowed to emerge under natural window lighting. *C. orae* pupae were kindly suppled by Dr. George Harvey of the Great Lakes Forest Center, while the *C. fumiferana* pupae were generously given by Dr. Gary Grant of the Forest Pest Management institute (Sault Ste. Marie, Ontario). Two-day-old insects were injected with a 1-μl solution of [^{14}C]acetate in phosphate buffer (pH 7); each insect received 18 nmol of acetate (~ 1 μCi). The needle was inserted between

the third and fifth abdominal segments and pushed towards the gland. Insects injected between 4 and 5 PM were left for 1–2 hr, after which the glands were either excised and extracted or the insects placed in a trapping chamber for 1 hr. Glands were extracted for 30 min with hexane containing 10% acetone and 0.005% butylated hydroxytoluene (BHT), and the extract was applied directly to TLC plates.

Trapping. Two-liter Erlenmeyer flasks were washed sequentially by soap, water, vinegar, and ether and then coated with 50 μl of 0.01 M tetradecanal in ether containing BHT to deactivate the glass surface. Groups of 20 insects were placed in the flasks, and air was pulled through a small hole in the top at a flow rate of 1 liter/min by the application of a vacuum to the exit tube. The exit tube extended almost to the bottom of the flask, and 0.2 g Porapak Q was held at the terminus by the glass wool inserted into a constriction in the tube. After trapping, the Porapak resin was extracted for 30 min with hexane, the extract concentrated and applied to TLC.

Chromatography. Samples were chromatographed on Machery-Nagel Silica-HN TLC plates either directly activated at 100°C for 60 min or sprayed with a solution of 5 g $AgNO_3$ in 85% ethanol before heat activation. TLC plates used for two-dimensional chromatography were prepared by spraying only the center of a plate with the $AgNO_3$ solution. All plates were developed for 60 min in hexane–diethyl ether–acetic acid (90:10:2). The TLC plates used for two-dimensional chromatography were dried for 5 min at 40°C after the first development and then rotated 90 degrees clockwise before the second development. Unlabeled standards were visualized by exposure to I_2 vapor, and radiolabel visualized by exposure of the plates to Kodak X-AR Omat film at −70°C for several weeks.

Enzyme Analysis. Acetate esterase activity was determined from the hydrolysis rate of 2.8 μM *E*-11-tetradecenyl acetate by 2 μg/ml gland homogenate in 3.5 ml of 0.05 M phosphate buffer (pH 8) as described (Morse and Meighen, 1984a). The alcohol product was converted into aldehyde by a brief reaction with 6.6 μg/ml horse liver alcohol dehydrogenase and 33 μM NAD^+. One-milliliter aliquots were then withdrawn from the reaction mixture and injected into 1 ml of phosphate buffer (pH 7) containing 10 μg *Vibrio harveyi* bacterial luciferase, 0.01 M hydroxylamine, and 50 μM FMN reduced with 1 mg of sodium dithionite. Light intensity is proportional to the amount of alcohol produced in the acetate esterase reaction and can be converted to picomoles by calibration with known concentrations of alcohol carried through the same procedure.

Alcohol oxidase activity was measured by the rate of oxidation of 2.8 μM *E*-11-tetradecenol catalyzed by 2 μg/ml of gland homogenate in pH 7 phosphate buffer. Aldehyde was measured directly by injection of 1.0-ml aliquots into 1.0 ml of phosphate buffer (pH 7.0) containing 10 μg bacterial luciferase, 0.01 M

hydroxylamine, and 50 μM FMN reduced with 1 mg of sodium dithionite. Light intensity is proportional to the amount of aldehyde produced in the alcohol oxidase reaction and can be converted into picomoles by calibration with known amounts of aldehyde carried through the same procedure.

Acetyltransferase activity was calculated from the rate of formation of labeled ester from 31 μM [^{3}H]acetyl-CoA and 10 μM *E*-11-tetradecenol by 10 μg/ml of gland homogenate in pH 7 phosphate buffer (Morse and Meighen, 1987a). After a 60-min reaction, the mixture was extracted with 1 ml hexane. Aliquots (50 μl) of the hexane extract were counted with 45% efficiency in 10 ml Econofluor, and the amount of ester formed was calculated from the specific radioactivity of the substrate (200 cpm/pmol).

RESULTS AND DISCUSSION

Resolution of the acetate-labeled glandular lipids of *C. orae* by two dimensional TLC on silica gel is given in Figure 1. The first dimension separates classes of lipids based on polarity, with the hydrocarbons being carried furthest by the solvent. In the second dimension, the chromatogram was impregnated with silver nitrate which retards unsaturated molecules (*Z* more than *E* isomers). Consequently, the saturated lipids (except for the fatty acids) are found on a diagonal line beginning at the origin, whereas the unsaturated molecules are located off the diagonal. The reason for the faster migration of the saturated fatty acids in the presence of silver nitrate is not known. The structural variations in the triglycerides arising from different combinations of saturated and unsaturated fatty acids with glycerol can be seen easily after resolution in the second dimension. The distribution of labeled glandular lipids from *C. orae* is identical to that found in *C. fumiferana* (Figure 2, lane 3).

The labeled ester in *C. orae*, which comigrates with tetradecenyl acetate, is composed primarily of the *E* isomer ($\geq$90%) with lower amounts of the *Z* isomer in approximately the same ratio as found in *C. fumiferana* for the *E*- and *Z*-11-tetradecenyl acetates. In radiolabeling studies in *C. fumiferana*, the tetradecenyl acetate esters had the highest turnover rate of all glandular lipids ($t_{\frac{1}{2}} \sim 6$ hr), consistent with the acetate ester being a storage precursor of the pheromone released from the insect during the calling period (Morse and Meighen, 1984b). A similar rate of turnover of the acetate ester was also observed for *C. orae* (data not given), consistent with the ester or a product thereof being released as the pheromone.

The volatile pheromones released from *C. orae* and *C. fumiferana* were analyzed after injection of labeled acetate into the insects and collection of the emitted volatiles on a Porapak Q filter. TLC analysis reveals that *C. orae* emits a blend of acetate ester and alcohol in approximately a 10:1 ratio as well as

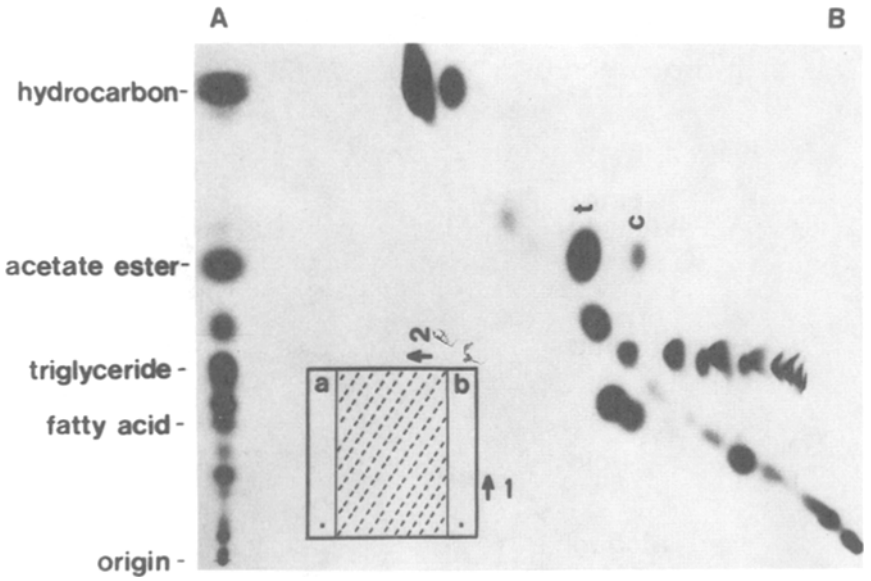

FIG. 1. Analyses of acetate-labeled lipids in gland extracts. Twenty *C. orae* female moths were labeled with [1-^{14}C]acetate for 2 hr and the gland extract applied to a TLC plate in both lanes A and B and chromatographed in the first dimension (↑1) as described in Materials and Methods. The TLC plate was rotated 90° clockwise after the first development to resolve in the second dimension (↑2) the saturated and unsaturated compounds in the B lane on the center of the impregnated silver nitrate plate (hatched area in schematic). The positions of migration of unlabeled standards including the *E*-11- (t) and *Z*-11-tetradecenyl acetate (c) are shown. Radiolabeled lipids in the gland extracts were visualized by autoradiography.

trace amounts of hydrocarbons (Figure 2, lane 1). In contrast, only the aldehyde pheromone, along with very low amounts of hydrocarbon, are released from *C. fumiferana* (lane 2). The nature and role of the hydrocarbon component of the budworm volatiles is unknown, although it is an intriguing possibility that they play some role in the pheromone blend. Although not yet shown in Lepidoptera, hydrocarbon pheromones are known to exist in other insects (Dillwith et al., 1981).

The amounts of labeled ester and alcohol from *C. orae* (20 insects) trapped on Porapak Q are greater than the amount of labeled aldehyde trapped from *C. fumiferana* (60 insects). However, a major proportion of the released pheromone from the latter species was bound to the glass container (lane 4), so that individuals of both species release approximately the same amount of labeled pheromone. In addition, small amounts of fatty acid and alcohol are also bound

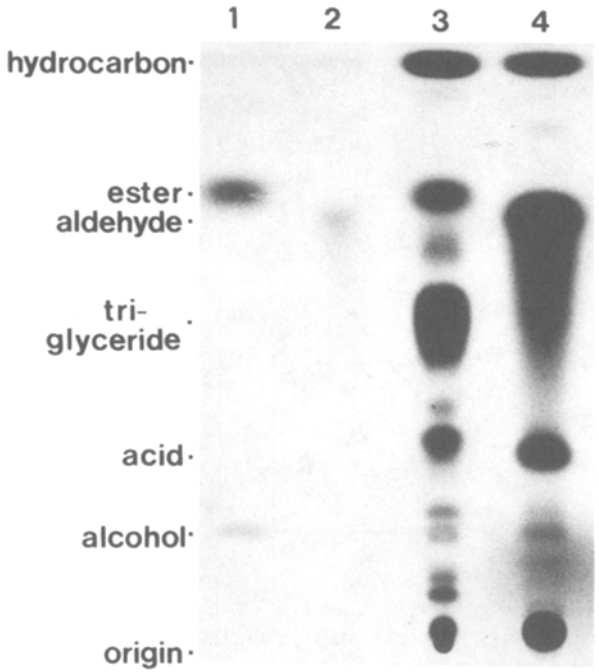

FIG. 2. Release of labeled pheromone from female budworm moths. Pheromone, released into an airstream, was trapped for a period of 1 hr on Porapak Q from insects injected with [1-^{14}C]acetate. Extracts of the Porapak Q traps containing labeled volatiles from 20 *C. orae* (lane 1) or from 60 *C. fumiferana* (lane 2) were analyzed by TLC and autoradiography. Insects were labeled between 5 and 6 PM, and volatiles trapped between 6 and 7 PM. The labeled lipids extracted from the glands of *C. fumiferana* after trapping (lane 3) and an ether wash of the trapping flask which contained this species (lane 4) are shown for comparison.

to the container. Figure 2 also shows that the labeled lipids extracted from the glands of *C. fumiferana* (lane 3) have the same lipid composition as the *C. orae* glands (Fig. 1A) and that the acetate ester precursor and not the aldehyde pheromone is present.

In *C. fumiferana*, the biosynthesis of the aldehyde pheromone arises via hydrolysis of the ester by an acetate esterase followed by oxidation of the alcohol product by an alcohol oxidase (Morse and Meighen, 1984a, 1986). An acetyl-CoA fatty alcohol acetyltransferase has been shown to be directly responsible for the synthesis of the acetate ester in this species (Morse and Meighen, 1987a).

These three enzymes were assayed in gland extracts from *C. orae* and the

levels compared to the activities found in *C. fumiferana* glands (Table 1). The extracts from the glands were prepared in an identical manner and assayed using the same solutions. Acetyltransferase levels, which vary with the age of the spruce budworm moths (Morse and Meighen, 1987a), were identical in extracts from *C. orae* and *C. fumiferana* moths at one, three, and five days after emergence from the pupae, indicating that the enzyme is under the same developmental regulation in both species. This result is also consistent with the comparable levels of acetate esters labeled in vivo in the glands of these two species (Figures 1 and 2). In contrast, the levels of enzymes involved in conversion of the acetate ester to aldehyde and, in particular, the alcohol oxidase, are lower in gland extracts from *C. orae*, consistent with its pheromone being a blend of the acetate ester and alcohol. The sixfold higher level of alcohol oxidase activity in *C. fumiferana* would account for the production and subsequent release of the blend of aldehydes used for its pheromone. The levels of esterase and oxidase activities in gland extracts of *C. orae* are very close to the levels obtained for the respective enzymes (1.4 pmol/min and 0.13 pmol/min per μg) in extracts of other body parts of *C. fumiferana* (Morse and Meighen, 1984a), possibly reflecting a basal level of metabolism of these compounds throughout the insect catalyzed by other enzymes with similar specificities.

The results are consistent with a mechanism in which the different levels of esterase and oxidase enzymes alter the relative amounts of ester, alcohol, and aldehyde, thus resulting in different pheromone blends of these compounds in *Choristoneura* budworm moths. All the metabolic steps leading to formation of the acetate ester and the regulation of its biosynthesis appear to be identical

TABLE 1. PHEROMONE ENZYME LEVELS IN GLANDS OF TWO *Choristoneura* SPECIES[a]

	Enzyme Activity				
			Acetyltransferase		
Insect	Oxidase	Esterase	Day 1	Day 3	Day 5
C. orae	0.24 ± 0.15	2.1 ± 1.0	0.04	0.25	0.13
C. fumiferana	1.3 ± 0.9	3.0 ± 2.5	0.05	0.25	0.11

[a] The glands from five groups of at least 10 female *C. orae* or *C. fumiferana* moths were extracted and the oxidase, esterase, and acetyl transferase activities measured in units of pmol product formed per min per μg of protein (see Materials and Methods). The acetyltransferase activity, which varies with the age of the adult moth, is shown for several different days after eclosion of the adults from the pupae. Oxidase and esterase activities, which do not vary with insect age, were averaged for all insects (age 1–7 days). Standard deviations are given for the oxidase and esterase activities in the table. For acetyltransferase activities, standard deviations are less than ±10% of the given values in all cases.

in the two budworm species, at least as reflected by the amounts of acetate ester in vivo and the levels of acetyltransferase in vitro. In *C. orae*, the activity of the alcohol oxidase is very low, and thus the acetate ester and alcohol are released as pheromone; in *C. fumiferana* the levels of esterase and, in particular, the oxidase are much higher, and the acetate ester is converted into aldehyde before release. Similar mechanisms may exist in other insect families to distinguish different species (Morse and Meighen, 1986). Gland extracts of *Heliothis* moths, which emit blends of unsaturated 14- and 16-carbon aldehydes, alcohols, and acetate esters, also contain the esterase and oxidase enzymes (Teal and Tumlinson, 1986). These authors have also suggested that these enzymes control the specific pheromone blend in this species (Tumlinson and Teal, 1987).

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IRIDOID GLYCOSIDE METABOLISM AND SEQUESTRATION BY *Poladryas minuta* (LEPIDOPTERA: NYMPHALIDAE) FEEDING ON *Penstemon virgatus* (SCROPHULARIACEAE)[1]

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Abstract—A bivoltine checkerspot butterfly, *Poladryas minuta*, is a *Penstemon* specialist, not known to utilize any other plant genus for oviposition and larval feeding. At several intermontane plains sites of central Colorado, the butterfly utilizes *Penstemon virgatus* as its sole host plant. Analysis of the host plant showed it to contain three cinnamyl-type catalpol esters (scutellarioside-II, globularin, globularicisin) and catalpol. The host plant contained an average of 10% dry weight iridoids, but some variation among individual plants and leaves within plants was noted. Field-collected butterflies contained 2.1–8.7% dry weight catalpol, but no other iridoids. Adults from larvae fed *P. virgatus* in the lab contained 4.2–9.0% dry weight catalpol and excreted large amounts of catalpol in the meconium. No catalpol was found in the larval frass. Larvae did not consume three alternate iridoid-containing host-plant species, and most eventually died rather than feed on the alternate plants. Larvae did consume small amounts of artificial diets containing the alternate species and *P. virgatus*, but most went into diapause and some died. Survival was good on artificial diet containing 10% dry weight of the iridoid esters from *P. virgatus*. Only catalpol was found in pupae and adults, but it was absent from the larval frass. The cinnamic-type acids expected from larval hydrolysis of the esters were not found in larval frass, pupae, or adults. These results are contrasted with those found for another checkerspot, *Euphydryas anicia*, which consumes a different host-plant species but was present at one of the same sites with *Poladryas minuta*.

[1] Paper 15 in the series "Chemistry of the Scrophulariaceae." Paper 14: Boros, C.M., Stermitz, F.R., and Harris, G.H. 1990. *J. Nat. Prod.* 53:72–80.

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Key Words—*Poladryas minuta*, Lepidoptera, Nymphalidae, checkerspot, *Penstemon virgatus*, *Penstemon secundiflorus*, Scrophulariaceae, iridoid glycosides, catalpol, sequestration, metabolism, herbivory.

INTRODUCTION

We have been studying natural lepidopteran populations that sequester iridoid glycosides as a result of specialization on species of the Scrophulariaceae. The work has involved (1) the checkerspot butterfly *Euphydryas anicia* on *Castilleja integra* and *Besseya plantaginea* at central Colorado montane sites (Stermitz et al., 1986; Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990), (2) another checkerspot, *Thessalia fulvia*, on *C. integra* in southern Colorado (D. Gardner and E. Mead, unpublished results), (3) a suggested Müllerian mimic pair (the geometrid moths *Meris alticola* and *Neoterpes graefiaria*) on *Penstemon* in Arizona (Stermitz et al., 1988); and (4) another geometrid (*Meris paradoxa*) and a noctuid moth (*Lepipolys perscripta*) on *Maurandya* in Arizona (C.M. Boros, unpublished results). Along with these chemically focused studies, we have chosen one of the systems (*E. anicia* on *Castilleja* and *Besseya* at Red Hill in central Colorado) for extensive behavioral investigations (Odendaal et al., 1988, 1989a,b). By combining the field behavioral and chemical data, we hope to better evaluate the place of iridoid sequestration in the ecology of the plant–insect interactions.

Focus of the present study was the checkerspot nymphalid butterfly *Poladryas minuta* (Edwards). There are a number of life-history differences between *P. minuta* and *E. anicia*, which suggested that a comparison of host plant iridoid content and insect iridoid sequestration patterns might be of interest. The *Euphydryas* larvae are essentially resource specialists and consume host plants from different genera and even different families as long as the hosts contain iridoid glucosides. Individual *Euphydryas* populations may, however, restrict themselves to one or a few among available iridoid-containing hosts. *Poladryas*, on the other hand is a strict *Penstemon* specialist and, although individual populations may utilize different *Penstemon* species, no other plant genus has yet been reported to serve as a host (Ferris and Brown, 1981; Scott, 1974, 1986). Throughout its range, *Euphydryas* is monovoltine, with immature larvae aestivating through the dry season in warmer climates and overwintering in more severe climates. *Poladryas*, even at near 10,000 feet in central Colorado, is at least bivoltine. Immature larvae of the late brood overwinter and consume young *Penstemon* in the spring, yielding adults in June to early July. Larvae from these adults feed on mature *Penstemon* and eventually give a second adult brood in late July and August. In some locations, there may be overlapping broods. We undertook the study to establish whether or not iridoids were

sequestered by *P. minuta*, and, if so, to quantitatively determine the relationships between plant and insect iridoid content.

METHODS AND MATERIALS

Organisms and Sites. Poladryas minuta (W.H. Edwards) occurs from Mexico and western Texas to Nebraska, Wyoming, Colorado, New Mexico, and west to California. The Texas populations are usually described as *P. minuta*, while those of Colorado have sometimes been considered as *P. arachne* (W.H. Edwards) (Ferris and Brown, 1981). The two taxa are considered by others (Scott, 1974) to be conspecific. Our populations are perhaps best described (Scott, 1986) as *P. minuta* ssp. *arachne*. The main study was conducted at Michigan Hill, a 2960-m site in Park County of central Colorado, which was previously described in detail (L'Empereur and Stermitz, 1990). Collections of butterflies also were made 8.6 miles south of Hartsel, Park County (off State Highway 9), 6.0 miles south of Buena Vista, Chaffee County (Mt. Princeton site), and 2 miles west of Buena Vista. Our collections were identified by Paul M. Opler, U.S. Fish and Wildlife Service, Fort Collins, Colorado.

At all sites, the only host on which eggs or larvae were found was *Penstemon virgatus* A. Gray, identified by Prof. D.M. Wilken, Department of Biology, Colorado State. A voucher sample (FRS282) is deposited in the Colorado State University herbarium. For feeding studies, *P. secundiflorus* Benth. (voucher sample FRS350, identified by D.M. Wilken) was collected at milepost 179 on U.S. Highway 285 south of Fairplay, Park County. *Castilleja integra* A. Gray and *B. plantaginea* (James) Rydb. (previously identified: L'Empereur and Stermitz, 1990) were collected at Michigan Hill.

Plant Extractions and Iridoid Analyses. For a large-scale extraction to identify the iridoid glycosides, a June 26, 1986, collection of *P. virgatus* (FRS251) from 7.1 miles west of Poncha Springs, Chaffee County, on U.S. Highway 50 was used. Plant material was dried at room temperature, powdered in a Waring blender and 186 g extracted without heating in 1000 ml MeOH for 48 hr. The mixture was filtered and the residue evaporated to leave a foamy residue. This was dissolved in 200 ml of H_2O and the solution extracted three times with 200-ml portions of Et_2O. The H_2O was evaporated to leave 32.6 g of residue, which was examined for iridoids by TLC (Si gel, $CHCl_3$–MeOH 65:35). The residue was dissolved in 300 ml of H_2O and extracted with 300 ml of 1-butanol to remove iridoids from the bulk of the sugars. TLC of the H_2O and 1-butanol layers indicated iridoids only in the 1-butanol, which was evaporated to leave 11 g of crude iridoids. A 50-mm-diameter flash chromatography column was packed with 15 cm of 60–200 mesh Si gel and 5 g of the crude iridoid residue separated using 200 ml each of 80:20, 70:30, 65:35, and 50:50

$CHCl_3$–MeOH, followed by 100% MeOH. Fractions (20 ml each) were examined by TLC, and those containing iridoids were further purified by preparative HPLC (Altex Ultrasphere-ODS reverse-phase column on a Beckman 331 system equipped with a 110B isocratic pump, Beckman injector, and a 1000-μl microliter sample loop; detection was via 254 nm absorption with a Beckman 163 variable wavelength detector). Individual iridoids were characterized by [^{1}H]NMR spectroscopy at 270 or 500 MHz.

To sample plant material on a site, three plants from Mt. Princeton, similar to those used in a fresh plant feeding study, were selected, and individual leaves from each plant were analyzed for iridoid content. An extraction scheme similar to that above was used, and the crude iridoid residue was analyzed by the typical quantitative GC procedure (L'Empereur and Stermitz, 1990). The plants were collected in midseason, but were of three different types. Plant 1 was a young plant, in bloom, whose leaves showed some evidence of larval feeding; plant 2 was somewhat similar, older, but still in bloom, and showed no evidence of insect damage; plant 3 was mature, large, undamaged, and beyond the blooming stage.

Plants of *P. secundiflorus* were extracted as above, the extract carried to the crude iridoid (butanol extraction) stage, and then analyzed qualitatively for iridoid content by TLC and [^{1}H]NMR spectroscopy.

Feeding Studies. Several hundred *Poladryas* eggs were collected on leaves of *Penstemon virgatus* at Mt. Princeton on June 7, 1987, and allowed to hatch in the laboratory. The larvae were placed in four Petri dishes and fed fresh *P. virgatus*. Three weeks later, four groups of 10 fourth-instar larvae each were placed in separate Petri dishes and given fresh *P. virgatus*, *P. secundiflorus*, *C. integra*, or *B. plantaginea*. Fresh plant material was given every other day until all larvae had died or entered diapause. One week later, three more groups of 15 larvae each were taken from a large group being maintained on *P. virgatus* and again fed each of the other plant species. Additional larvae on *P. virgatus* were held until pupation. Ten pupae were removed for chemical analysis, while others were placed in small jars, lined with filter paper to catch meconium emitted upon eclosion, and the emergent adults were saved for analysis.

Artificial diets were prepared (Bowers, 1983; L'Empereur, 1989) that contained 25% by weight dried, powdered *P. virgatus* or 10%, 25%, and 50% of *P. secundiflorus* or *C. integra* or *B. plantaginea*. Another diet was prepared that contained 10% dry weight of a mixture of the catalpol esters found in *P. virgatus*. Diets were refrigerated until use. Larvae were hatched from eggs obtained from three adult females collected at the Mt. Princeton site and three collected at the site west of Buena Vista on June 21, 1988. Each female laid an average of 100 eggs, most of which hatched one week later. Larvae were raised on fresh *P. virgatus* for 10 days, then on the 25% *P. virgatus* artificial diet for three days until they began consumption. Groups of 10 larvae each were then

placed on the artificial diets containing the other plant species and the catalpol esters, while a group of about 100 larvae were continued on the *P. virgatus* diet. Feeding was continued until all larvae had entered diapause, died, or pupated.

Quantitative iridoid analyses of individual late-instar larvae, pupae, emitted meconium, and adults were performed by GC of the trimethylsilyl ethers as previously described (Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990). Because many larvae on artificial diets went into diapause at an early stage, they were pooled according to diet type before analysis.

In a study of metabolism of the plant catalpol esters, five batches of frass, obtained from larvae raised on the ester-containing diet were dried, weighed, and extracted for 24 hr in 3 ml MeOH. Frass from larvae raised on fresh *P. virgatus* was also extracted. An emerged adult male and its emitted meconium were similarly treated. The MeOH extract was filtered, evaporated to dryness, taken up in 4.00 ml MeOH, and a 2.00-ml aliquot transferred to a 1-dram screw-cap vial for analysis. A standard solution of the following acids was prepared: *p*-hydroxybenzoic acid (0.23 mg/ml), cinnamic acid (0.023 mg/ml), *p*-coumaric acid (0.045 mg/ml), and 3,4,5-trimethoxycinnamic acid (0.054 mg/ml). The *p*-hydroxybenzoic acid is an hydrolysis product of methyl paraben, a preservative in the artificial diet, while the 3,4,5-trimethoxycinnamic acid was used as an internal standard. The two other acids are expected hydrolysis products of the catalpol esters in *P. virgatus* (see below). The acids were analyzed by hplc by injecting 20 μl onto an Altex Ultrasphere ODS column (25 cm $\times$ 15 mm). The mobile phase was 60% MeOH in 1 N HCOOH at a flowrate of 1.5 ml/min. Detection was by UV absorption at 254 nm. Under these conditions the acids eluted as follows: *p*-hydroxybenzoic acid at R_t = 2.78 min; *p*-coumaric acid at R_t = 3.26 min; 3,4,5-trimethoxycinnamic acid at R_t = 4.82 min; and cinnamic acid at R_t = 6.62 min.

RESULTS

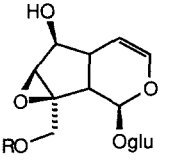
Poladryas minuta *Life History Observations*. Studies were carried out at Michigan Hill, south of Hartsel, and at Mt. Princeton rather than a single site since populations of butterflies at a single site were highly variable from year to year and even seasonably among broods. The host plant, *Penstemon virgatus*, was present at all sites in all years.

C. integra, an iridoid-containing host for *E. anicia*, occurs at Michigan Hill, south of Hartsel and at the Mt. Princeton site. No *Poladryas* eggs or larvae were ever encountered on *C. integra* at any of the sites. The Mt. Princeton site contains *Penstemon secundiflorus* in abundance similar to that of *P. virgatus*, but no eggs or larvae were ever found on *P. secundiflorus* even though extensive

searching was done. For example, on July 2, 1987, a search of 60 *P. virgatus* plants revealed 19 with egg masses (17–30 eggs per mass). No egg masses were found on 19 of 19 *P. secundiflorus* interspersed with the *P. virgatus*. At Mt. Princeton, *P. secundiflorus* blooms in July, earlier than *P. virgatus*, and also senesces earlier, usually having disappeared by the end of July. On the other hand, *P. virgatus* usually reaches full bloom only by the end of July and can be found until frost in September.

Six captured females each laid approximately 100 eggs on *P. virgatus* in the course of three days in the laboratory. Egg hatch in the laboratory was approximately seven days after oviposition.

Penstemon Analyses. The crude iridoid fraction from the large scale isolation showed four *p*-anisaldehyde positive spots by TLC: R_f = 0.23 (brown; UV inactive), 0.23–0.25 (pink; UV absorbing), 0.42 (brown; UV absorbing), and 0.53 (brown; UV absorbing). Flash chromatography gave the R_f = 0.53 compound in fraction 4, a mixture of the R_f = 0.53 and 0.42 compounds in fractions 5–10, all four compounds in fractions 11–12, a majority of the pink spraying compound in fraction 13, and mostly the R_f = 0.23 brown-spraying compound in fractions 15–25. The [^{1}H]NMR spectra (270 MHz) and TLC R_f values identified the R_f = 0.23 compound as catalpol, **1** (Scheme 1), and the R_f = 0.23–0.25 compound as the common phenylpropanoid glycoside verbacoside, both in comparison with previous isolates. Reverse-phase TLC analysis (KC-18, H_2O–MeOH 60:40 + 0.15 mg NaCl per 10 ml) of the fraction 5–10 mixture showed three components (R_f = 0.10, 0.16, and 0.32) rather than two. HPLC of the mixture was performed with elution by 67:33 H_2O–MeOH at 5.0 ml/min to yield pure fractions at R_t = 7.5, 19.0, and 32.0 min. These were shown to be scuttelarioside-II, **2,** globularicisin, **3,** and globularin (also known as scuttelarioside-I), **4,** by 270 MHz [^{1}H]NMR analysis in comparison with literature data for **2** (Weinges et al., 1975) and **4** (Fauer et al., 1987) and by 500 MHz ^{1}H-^{1}H 2D COSY NMR for **3.** The spectra are available in a thesis (L'Empereur, 1989).



1: R = H; catalpol
2: R = *trans*-4-hydroxycinnamoyl; scutellarioside-II
3: R = *cis*-cinnamoyl; globularicisin
4: R = *trans*-cinnamoyl; globularin

SCHEME 1.

Quantitative individual plant and leaf analyses (Table 1) showed the same iridoids as in the large-scale isolation. Globularicisin could not be quantified exactly because we were unable to obtain sufficient pure sample for standard injection. Qualitatively, the GC peak was $\frac{1}{3}$ to $\frac{1}{2}$ the height of that for globularin, except in plant 1, where it was slightly higher.

Poladryas minuta *Analyses*. Adult *P. minuta* were collected from early and/or late broods at Michigan Hill in 1984–1986, from south of Hartsel in 1985, and from Mt. Princeton in 1985–1988 and examined qualitatively by TLC for iridoid presence. None of the esters **2–4** were found, and the only iridoid detected was catalpol. Quantitative analyses on field-collected adults from Michigan Hill also showed only catalpol (Table 2), and this was true for both sexes and June and late July broods. Insufficient numbers of individuals from all groups did not allow for useful statistical analyses across the various categories, and the population variability did not allow for an intensive study of early vs. late brood differences. Female dry weight was about twice that of male dry weight, as is common for checkerspot butterflies. The total range of catalpol concentration for all 36 field-captured butterflies was 2.1–8.7% of the dry weight, with the males containing about one-half the catalpol of females and hence a similar dry weight iridoid concentration.

Catalpol was also the only iridoid found in the adult butterflies raised in the laboratory on fresh *P. virgatus* (Table 2). Upon eclosion, males emitted about the same quantity of catalpol in the meconium as they maintained, while females emitted about one-third of the quantity kept (Table 3). Frass was collected during the larval feeding phase and was found to be devoid of catalpol. Three adult lab-raised females were separated into abdomen, thorax plus legs, and wing parts and each showed appreciable catalpol content (Table 4).

P. minuta *Viability in Lab Feeding Studies*. Viability on fresh *Penstemon*

TABLE 1. IRIDOID ANALYSES OF INDIVIDUAL LEAVES OF THREE *Penstemon virgatus* PLANTS

	Plant 1		Plant 2				Plant 3				
Iridoid (%)	Leaf 1	Leaf 2	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Average
Catalpol	3.0	4.0	0.9	0.6	0.7	0.9	0.9	0.6	1.8	2.2	1.6
Globularin	0.3	0.1	1.3	5.0	4.0	7.6	4.9	2.9	2.4	2.9	3.1
Scutellarioside-II	7.0	7.0	4.2	3.1	3.3	3.7	8.7	1.9	8.7	2.9	5.6
Total iridoids[a]	10.3	11.1	6.4	8.7	8.0	12.2	14.5	5.4	12.9	13.2	10.3

[a] Quantities could not be accurately determined for globularicisin, but it was estimated by GC peak height relative to globularin that the content was 1/3 to 1/2 that of globularin in plants 2 and 3 and slightly more than globularin in plant 1.

TABLE 2. CATALPOL (**1**) CONTENT OF FIELD-CAUGHT AND LAB-RAISED ADULT *Poladryas minuta*

		N	Dry wt. (mg) mean ± SD *(range)*	**1** (mg) mean ± SD *(range)*	% Dry wt. **1** mean ± SD *(range)*
Field caught (Michigan Hill site)					
Females	6/85	5	**28.8** ± 2.8 *(25.6–31.9)*	**1.37** ± 0.29 *(0.90–1.68)*	**4.7** ± 0.8 *(3.5–5.5)*
	7/86	4	**22.7** ± 5.7 *(18.0–30.8)*	**0.88** ± 0.23 *(0.69–1.19)*	**3.9** ± 0.5 *(3.3–4.6)*
Males	6/85	15	**14.2** ± 2.8 *(8.8–20.0)*	**0.54** ± 0.12 *(0.41–0.78)*	**3.8** ± 0.7 *(2.7–4.9)*
	7/85	5	**12.4** ± 0.8 *(11.4–13.7)*	**0.34** ± 0.09 *(0.27–0.50)*	**2.8** ± 0.8 *(2.2–4.2)*
	7/86	8	**13.2** ± 1.4 *(11.5–14.9)*	**0.49** ± 0.04 *(0.45–0.57)*	**3.7** ± 0.3 *(3.2–4.2)*
Lab Raised (Mt. Princeton site)					
Females	8/87	10	**25.4** ± 5.1 *(15.9–31.2)*	**1.61** ± 0.54 *(1.02–2.73)*	**6.3** ± 1.3 *(4.2–8.7)*
Males	8/87	10	**16.2** ± 1.6 *(14.0–18.2)*	**0.94** ± 0.32 *(0.59–1.65)*	**6.1** ± 1.8 *(4.2–9.0)*
Pupae	8/87	10	**25.4** ± 5.4 *(16.8–33.3)*	**1.88** ± 0.72 *(0.83–3.07)*	**7.3** ± 2.2 *(3.2–9.9)*

TABLE 3. CATALPOL (**1**) CONTENT IN LAB-RAISED MALE AND FEMALE *Poladryas minuta* ADULTS AND EMITTED MECONIUM

	N	Dry wt. (mg) mean ± SD	**1** (mg) mean ± SD	% Dry wt. **1** mean ± SD
Males	10	**16.2** ± 1.6	**0.94** ± 0.32	**6.1** ± 1.8
Meconium	10	N/A	**0.81** ± 0.39	**5.0** ± 2.3
Total catalpol			**1.75** ± 0.44	**10.8** ± 2.4
Females	10	**25.4** ± 5.1	**1.61** ± 0.54	**6.3** ± 1.3
Meconium	10	N/A	**0.51** ± 0.25	**2.1** ± 1.2
Total Catalpol			**2.12** ± 0.60	**8.4** ± 1.8

TABLE 4. CATALPOL (1) CONTENT OF BODY PARTS FROM THREE INDIVIDUAL FEMALE *Poladryas minuta*

Part	Dry wt. (mg)	1 (mg)	1 (% dry wt.)
Female 1			
Thorax	10.1	0.51	5.1
Abdomen	11.1	0.25	2.2
Wings	4.5	0.14	3.1
Female 2			
Thorax	8.9	0.58	6.5
Abdomen	12.7	0.56	4.4
Wings	4.6	0.24	5.2
Female 3			
Thorax	11.0	0.37	3.3
Abdomen	15.9	0.77	4.9
Wings	5.0	0.24	4.9

virgatus was high, but almost nil on fresh *P. secundiflorus*, *B. plantagineae*, and *C. integra* (Table 5). Larvae essentially did not consume plants other than *P. virgatus* except for a little *B. plantaginea*. Lack of consumption was indicated by lack of damage to plants and an absence of frass. Although larvae consumed the artificial diets containing dried plant material from each of the

TABLE 5. VIABILITY OF *Poladryas minuta* RAISED ON VARIOUS DIETS

	Pupated	Diapaused	Died
Fresh Plant			
P. virgatus	37	2	1
P. secundiflorus	0	1	44
B. plantaginea	0	11	34
C. integra	0	1	44
Artificial diet (AD)			
P. virgatus/fresh	26	13	1
AD	0	23	7
AD + *P. virgatus*	1	21	18
AD + *P. secundiflorus*	1	39	0
AD + *B. plantaginea*	1	37	2
AD + *C. integra*	1	37	2

species, with a continual production of small amounts of frass, they almost all entered diapause prior to maturity (Table 5). One larva from each group matured sufficiently to pupate, but the pupae were deformed and only two eclosed, yielding deformed adults. Larvae did not distinguish among the 10%, 25%, and 50% plant diets and hence data for all groups are combined in Table 5.

Metabolism of Iridoids. In contrast to the case with artificial diets containing dried plant material, viability was much better with the diet containing 10% dry weight of ester iridoids **2–4.** Here, six larvae went into diapause before reaching maturity, but four formed pupae. Two of the pupae were sacrificed for analysis and two were kept until the adults eclosed, which were also analyzed. The pupae and adults contained catalpol, but none of the esters **2–4.** The diet itself was analyzed at the end of the feeding study and was shown to have maintained the esters, but to contain no catalpol. Larval frass collected during the feeding study did not contain *trans*-cinnamic acid, *cis*-cinnamic acid, or *p*-coumaric acid expected from hydrolysis of **2, 3,** or **4,** respectively. Frass did contain *p*-hydroxybenzoic acid resulting from larval hydrolysis of the methyl paraben present as a preservative in the artificial diet (Gardner and Stermitz, 1988). The frass contained no **2, 3,** or **4.** Traces of catalpol, which are not easily detectable by HPLC, were, however, detected by TLC and GC. The adult butterflies and the diapaused larvae were also analyzed for **2–4,** but none was found.

DISCUSSION

At the sites investigated, *Poladryas minuta* larvae have been found to consume *Penstemon virgatus*, which contains mostly cinnamic acid esters of catalpol, to hydrolyze the esters, and sequester the resultant catalpol. Thus, it shows some similarity to *E. anicia* on *B. plantaginea* (Gardner and Stermitz, 1988; L'Empereur and Stermitz, 1990), where benzoic acid esters of catalpol were hydrolyzed by larvae. In addition to being cinnamic acid rather than benzoic acid esters, those in *P. virgatus* are C-10 esters rather than the C-6 esters found in *B. plantaginea*. The insects may well possess a general esterase since larvae of both species also hydrolyzed methyl paraben from the artificial diet to *p*-hydroxybenzoic acid. Unlike the case of *E. anicia*, where the resultant benzoic acids were excreted in the frass, the cinnamic acids expected from hydrolysis of **2–4** were not found in frass, diapaused larvae, or adult butterflies. *P. minuta* must further metabolize the cinnamic acids. Epoxidation, diol formation, and/or transesterification (Mullin, 1988) are possibilities to be investigated.

The total iridoid content of *P. virgatus* and *B. plantaginea* (the major host plant of *E. anicia* at Michigan Hill) was similar, averaging around 10% dry weight, and hence both butterflies appear to have a similar resource at this site in terms of iridoids. They also exploit the sources similarly, with field-caught

adults of *Poladryas* containing 2.2–5.5% iridoids ($N = 36$), while those of *E. anicia* at the same site had 0.5–4.3% ($N = 31$) (L'Empereur and Stermitz, 1989). The *Poladryas* population did, however, lack the low end iridoid concentrations, which we have also found in other *E. anicia* populations: 0.8–9.1% ($N = 116$) at Red Hill and 0.7–10.4% ($N = 40$) at Cumberland Pass. *Poladryas* adults whose larvae were lab-raised on fresh host plant showed 4.2–9.0% iridoids ($N = 20$), and this suggests a greater potential for iridoid sequestration than is exhibited in the field.

The iridoid material balance was quite good with *Poladryas* on its host plant, with the average plant iridoid content (Table 1) reasonably matching the sum of the meconium and adult butterfly content (Table 3). This had not been the case with *E. anicia* on *B. plantaginea* (L'Empereur and Stermitz, 1990), where there was considerably less catalpol in the adults and meconium than expected based upon host plant and larval content. There we had suggested possible catalpol metabolism in the pupal stage.

The attempted feeding of alternate, iridoid-containing host plants to *Poladryas* larvae (Table 5) presented additional evidence for the monophagy of the genus. It was suggested (Scott, 1974) that this is due to a difference in leaf hardness and that larvae will consume tender new leaves of *Penstemon secundiflorus*. The artificial diet studies confirm that larvae will indeed consume material containing a variety of host-plant material, but the experiment did not really serve its intended purpose since larvae did as poorly on diet incorporating the *P. virgatus* as they did on diet with alternate host material. Reasons for this are unknown, but larvae did do reasonably well on the same diet containing a host plant-related concentration of iridoid esters.

The iridoid concentrations (percent dry weight) of catalpol in the three insect parts (thorax plus legs, abdomen, wings; Table 4) were relatively comparable considering the individual butterfly variation. Iridoid content of the wings represented less than 20% of the total butterfly iridoid load, mostly because of the lower dry weight of the wings than other body parts. This is to be contrasted with the case of cardenolide sequestration in monarch butterflies (Brower et al., 1988), where 41% of total cardenolides were found in the wings.

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ATTRACTION OF *Cacoecimorpha pronubana* MALE MOTHS TO SYNTHETIC SEX PHEROMONE BLENDS IN THE WIND TUNNEL

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Abstract—Attraction of *Cacoecimorpha pronubana* male moths to blends of four sex pheromone components was studied in the wind tunnel. Z11-14:Ac alone did not elicit upwind flight, admixture of 10% Z11-14:OH or more led to successful attraction of males to the source. Attractivity of these binary blends was further enhanced by addition of E11-14:Ac or Z9-14:Ac; the optimum was a blend of Z11-14:Ac, Z11-14:OH, E11-14:Ac, and Z9-14:Ac at the proportion of 100:30:3:3. In ternary and quaternary blends, the effect of Z11-14:OH and E11-14:Ac was strongest at 30% and 3%, respectively. By contrast, Z9-14:Ac increased behavioral responses over a wide range.

Key Words—*Cacoecimorpha pronubana* Hbn., Lepidoptera, Tortricidae, sex pheromone components, wind tunnel, attraction, pheromonal behavior.

INTRODUCTION

Female effluvia of the carnation tortrix, *Cacoecimorpha pronubana* contain Z-11-tetradecenyl acetate (Z11-14:Ac), E-11-tetradecenyl acetate (E11-14:Ac), Z-9-tetradecenyl acetate (Z9-14:Ac), tetradecyl acetate (14:Ac) and Z-11-tetradecen-1-ol (Z11-14:OH) at an average blend proportion of 82:5:1:5:7. Individual females emit these compounds in constant proportions. Release ratios of the Δ11-components Z11-14:Ac, E11-14:Ac, and Z11-14:OH are similar

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for different females, but release ratios of Z9–14 : Ac relative to the Δ11-components show strong interindividual variation (Witzgall and Frérot, 1989).

The behavioral activity of Z11–14 : Ac, E11–14 : Ac, and Z11–14 : OH in *C. pronubana* males has been established in olfactometer and field trapping tests (Descoins et al., 1985). However, the presence of receptor cells specific not only for these three components, but also for Z9–14 : Ac was suggested by Priesner (1979).

An account is presented here of the wind-tunnel responses of *C. pronubana* male moths to binary, ternary, and quaternary blends of Z11–14 : Ac, E11–14 : Ac, Z9–14 : Ac, and Z11–14 : OH.

METHODS AND MATERIALS

Insects. Insects were reared on a semiartificial diet. Sexes were separated as pupae and kept in different rooms in plastic containers (200 ml) under a 14 : 10 light–dark photoperiod at 20°C. Preliminary tests revealed a maximal activity of day-active *C. pronubana* males, 1–4 hr after the onset of the photophase. All tests were conducted with 3- to 4-day-old males during this period.

Wind Tunnel. The wind tunnel (Frérot et al., 1989a) was made of clear Plexiglas (200 cm long, 70 cm high, 90 cm wide). Air was pulled from outside the building through the tunnel at a constant speed of 30 cm/sec. Incoming air was filtered with three layers of active charcoal and heated to 20–22°C; pheromone-laden air was blown out of the building. The airflow in the tunnel was regularly visualized with incense sticks.

Test Chemicals. Isomeric purity of chemicals was ≥99.9%. Hexane solutions of the test compounds were applied to filter paper (1.5 × 1.5 cm) one day before testing. During tests, the filter paper was held by a disposable syringe needle at the top of a thin wire in the center of the tunnel, 10 cm from the upwind end.

Experimental Procedure. Z11–14 : Ac was tested singly; for binary and ternary blends, 1, 3, 10, 30, and 100 ng of Z9–14 : Ac, E11–14 : Ac, and Z11–14 : OH were added to 100 ng of Z11–14 : Ac. For blends comprising all four chemicals, 0.1, 0.3, 1, 3, and 10 ng of the minor components were added to 10 ng of Z11–14 : Ac. Each blend was tested on three different days with 20, 20, and 30 males ($N = 70$). Five males were transferred to wire cages ca. 1 hr before tests. A test cage (10 × 5 × 5 cm) was placed 20 cm from the downwind end of the tunnel at 30 cm from the floor. Before opening the cage, males were exposed to the test chemicals during 1 min. Behavioral responses such as wing-fanning before taking off, flying upwind, landing on the source, as well as duration of upwind flights from the holding cage to the source were recorded for 5 min.

The number of males arriving at the source was transformed to $\log(x + 1)$ and submitted to an analysis of variance, followed by a Tukey test ($P = 0.05$). Duration of upwind orientation flights was analyzed by a Kruskal-Wallis test.

RESULTS

Response to Binary Blends. Of all binary combinations tested, only blends of Z11–14 : Ac containing at least 10% of Z11–14 : OH elicited upwind orientation flights of *C. pronubana* males. The number of arrivals on the source increased with alcohol dose (Figures 1 and 2). At 100 ng Z11–14 : Ac and 100 ng Z11–14 : OH, 26% of males landed on the source and another 23% flew upwind, but did not reach the source. At 100 ng, Z11–14 : Ac alone did not induce upwind flights.

Response to Ternary Blends. Attraction of males to ternary blends is shown in Figure 2. Only blends containing both Z11–14 : Ac and Z11–14 : OH were attractive. The response pattern in Figure 2 shows a well-defined maximum for E11–14 : Ac at 1–3% with 10–100% Z11–14 : OH, but admixture of 1–30% Z9–14 : Ac elicited best attraction at 100% Z11–14 : OH. Significantly more males landed on blends containing 100 ng Z11–14 : Ac, 100 ng Z11–14 : OH, and 1–30 ng Z9–14 : Ac than on 100 ng Z11–14 : Ac and 100 ng Z11–14 : OH alone (Figures 1 and 2).

Response to Quaternary Blends. Quaternary blends of 100 ng Z11–14 : Ac, 30 or 100 ng Z11–14 : OH, 3 ng E11–14 : Ac, and 3 ng Z9–14 : Ac elicited significantly more wing-fanning during activation in the holding cage than the corresponding ternary blends. Even at an alcohol dose of only 1 ng, 24% males flew upwind, 4% of which landed on the source (Figure 1).

The number of males landing on a quaternary blend of 100 ng Z11–14 : Ac, 3 ng E11–14 : Ac, 3 ng Z9–14 : Ac, and 30 ng Z11–14 : OH and on a ternary blend of 100 ng Z11–14 : Ac, 3 ng E11–14 : Ac, and 30 ng Z11–14 : OH (Figure 1) were not statistically different. Tests with all four components were then conducted at a dose of 10 ng Z11–14 : Ac. At this level, a ternary combination with 3 ng Z11–14 : OH and 0.3 ng E11–14 : Ac attracted only 27% of the males tested ($N = 70$).

The percentage of males landing on quaternary blend sources containing 10 ng Z11–14 : Ac is shown in Figure 3. With these blends, the synergistic effect of Z11–14 : OH showed a well-defined maximum at 30%. At the nonoptimal alcohol doses (1 and 10 ng), most males were attracted to blends containing 3% E11–14 : Ac. By contrast, as observed with the ternary blend combinations (Figure 2), an amount of 1–30% Z9–14 : Ac in the blend induced similar response levels (Figure 3). Attraction was best (69%) at a blend of 10 ng Z11–14 : Ac, 3 ng Z11–14 : OH, 0.3 ng E11–14 : Ac, and 0.3 ng Z9–14 : Ac.

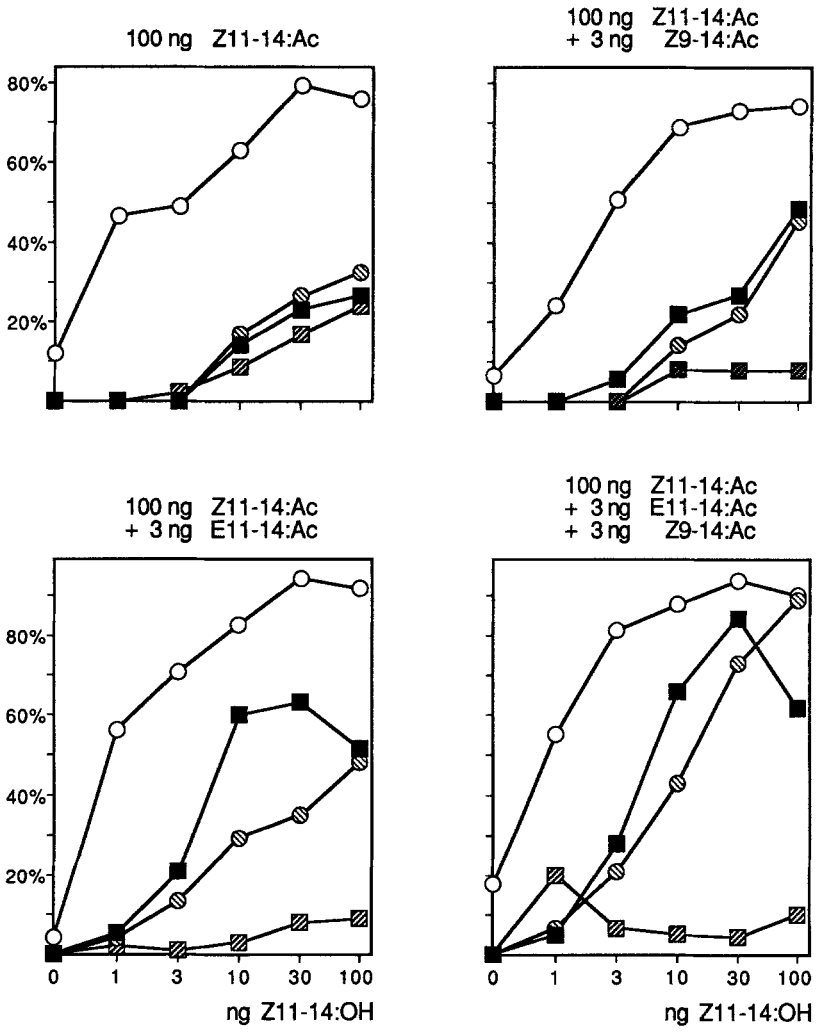

FIG. 1. Behavioral responses of male *C. pronubana* ($N = 70$) to binary, ternary, and quaternary blends of sex pheromone components at varying proportions of Z11-14:OH. Open circles: take-off from cage; hatched circles: wing-fanning in holding cage before take-off; hatched squares: unsuccessful orientation flights; solid squares: upwind orientation flights followed by landing on source.

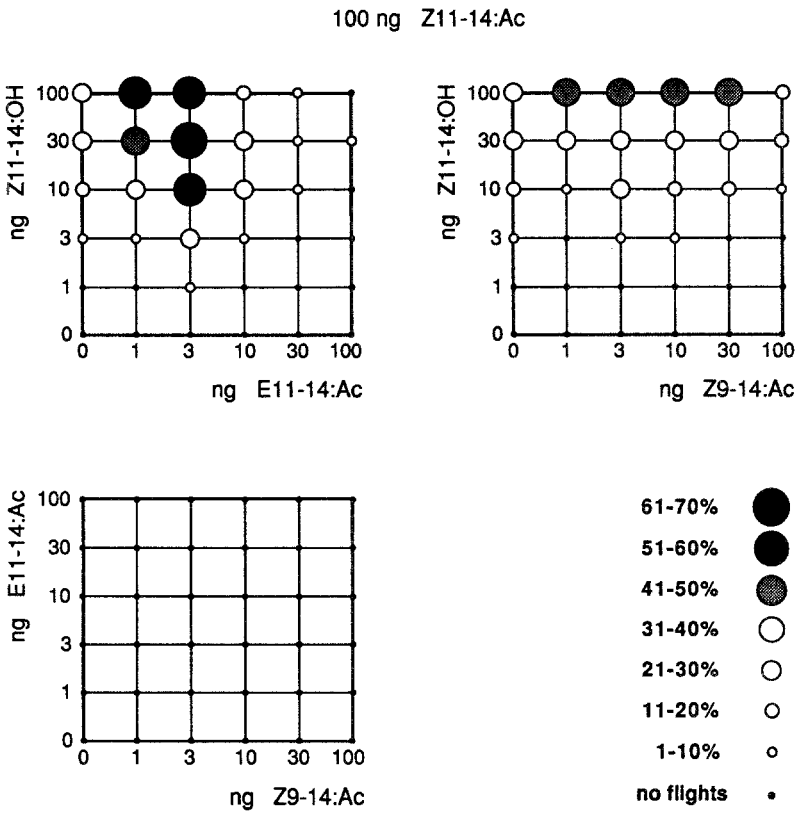

FIG. 2. Attraction (upwind flight followed by landing) of male *C. pronubana* ($N = 70$) towards ternary and binary blends of sex pheromone components. The amount of Z11-14:Ac was constant at 100 ng. Size and shading of circles indicates percent successful upwind flights.

Behavioral Observations. Visual observations of flight behavior towards quaternary and ternary mixtures showed that blends with large amounts of E11-14:Ac (>10%) elicited "erratic" flight tracks with pronounced crosswind excursions (zigzags). By contrast, high doses of Z11-14:OH induced strong reduction of both crosswind excursions and ground speed; the insects sometimes "hovered" for several seconds without advancing towards the source. Consequently, flight duration towards binary and ternary blends containing 100 ng Z11-14:OH was significantly higher than with blends of 10 ng Z11-14:OH (Table 1). Generally, flight tracks to blends near the optimum were more direct than to suboptimal blends.

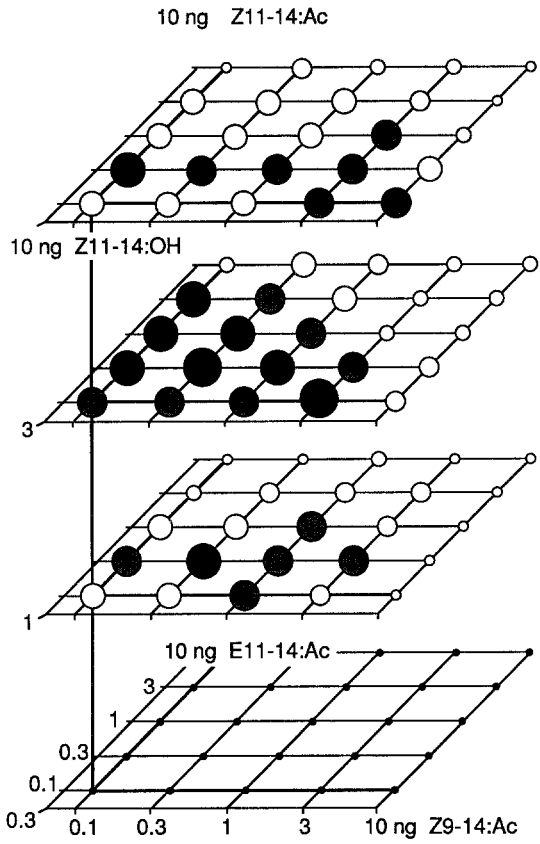

FIG. 3. Attraction of male *C. pronubana* (N = 70) to quaternary blends of sex pheromone components. The amount of Z11–14 : Ac was constant at 10 ng. Size and shading of circles indicates percent successful upwind flights (see Figure 2).

DISCUSSION

The sex pheromone of the carnation tortrix, *C. pronubana*, consists of Z11–14 : Ac, Z11–14 : OH, *E*11–14 : Ac, and Z9–14 : Ac (Descoins et al., 1985, Witzgall and Frérot, 1989; this study). Of the other compounds present in the female effluvia (Witzgall and Frérot, 1989, Frérot et al., 1989b), only tetradecyl acetate (14 : Ac) has been tested in the wind tunnel and field. However, attractivity of ternary (Z11–14 : Ac, Z11–14 : OH, *E*11–14 : Ac, or Z9–14 : Ac) and quaternary blends could not be augmented by admixture of 14 : Ac (Frérot, unpublished).

Single components of the *C. pronubana* sex pheromone were not attractive at doses of 10 or 100 ng on filter paper. Significantly more males responded by

upwind flight to ternary blends (100 ng Z11–14 : Ac, 10–100 ng Z11–14 : OH, and 3 ng *E*11–14 : Ac or 100 ng Z11–14 : Ac, 100 ng Z11–14 : OH, and 1–30 ng Z9–14 : Ac) than to corresponding binary blends of 100 ng Z11–14 : Ac with 10–100 ng alcohol (Figures 1 and 2). Attraction was best with a 100 : 30 : 3 : 3 blend of all four components (Figure 3).

The behavioral threshold for upwind orientation flight to the complete blend was thus lower by comparison with incomplete blends. Behavioral studies on *Grapholita molesta* (Linn and Roelofs, 1983), *Trichoplusia ni* (Linn et al., 1984, 1988), *Cydia pomonella* (Arn et al., 1985), *Pectinophora gossypiella* (Linn and Roelofs, 1985), *Argyrotaenia velutinana* (Linn et al., 1986), *Eupoecilia ambiguella* (Arn et al., 1986b), or *Spodoptera litura* (Kawasaki, 1986) in the wind tunnel and on *Adoxophyes orana* (Guerin et al., 1986) and *Pennisetia hyaeliformis* (Priesner et al., 1986) in the field have provided experimental evidence that minor components function to enhance male sensitivity.

Furthermore, these studies show that "individual minor components do not function to trigger specific behaviors in the response sequence, but rather that the full blend acts as a unit in the male flight response and courtship sequence" (Linn et al., 1986). However, differences among the components of the full pheromone blend exist insofar as in many lepidopteran species one or two components are sufficient for attraction of males (see Arn et al., 1986a), whereas the admixture of further compounds, unattractive in themselves, only enhance the attractivity of the "major" compound(s). In *C. pronubana*, presence of both Z11–14 : Ac and Z11–14 : OH is mandatory to induce orientation flights followed by landing on the source. Even a ternary blend of Z11–14 : Ac, *E*11–14 : Ac, and Z9–14 : Ac is not attractive in the wind tunnel. The latter two components only enhance expression of the behavioral sequence, but do not elicit attraction responses by themselves.

The behavioral observations on *C. pronubana* orientation flights in the wind tunnel indicate qualitative differences among individual unsaturated components of the female effluvium: High amounts of Z11–14 : OH prolong flight duration (Table 1) and clearly reduce the amplitudes of lateral inflight excursions. On the other hand, large amounts of *E*11–14 : Ac result in erratic flight patterns with wide and rapid crosswind zigzags. Modification of flight tracks by minor pheromone gland components has been demonstrated in *Euxoa ochrogaster* (Palaniswamy et al., 1983) and in *Grapholita molesta* (Willis and Baker, 1988).

Conspicuously, males exhibited highest response levels to blends containing 30% Z11–14 : OH and 3% *E*11–14 : Ac. By contrast, amounts ranging from 1 to 30% Z9–14 : Ac in the blend enhanced upwind flight responses (Figures 2 and 3). This finding is particularly noteworthy as the interindividual variation of release ratios of the Δ11-components (Z11–14 : Ac, *E*11–14 : Ac, Z11–14 : OH) is small in *C. pronubana* females, whereas release ratios between Z9–

TABLE 1. DURATION OF SUCCESSFUL ORIENTATION FLIGHTS[a] OF MALE *C. pronubana* TO BLENDS CONTAINING 100 ng Z11-14:Ac; 10 ng, 30 ng, AND 100 ng Z11-14:OH AND VARYING AMOUNTS (1-100 ng) OF *E*11-14:Ac AND Z9-14:Ac

Z11-14:OH (ng)	100 ng Z11-14:Ac		100 ng Z11-14:Ac, 1-100 ng Z9-14:Ac		100 ng Z11-14:Ac, 1-100 ng *E*11-14:Ac	
	Sec ± SD	*N*	Sec ± SD	*N*	Sec ± SD	*N*
10	21 ± 12 a[b]	14	20 ± 9 a	19	23 ± 11 a	32
30	26 ± 11 ab	23	24 ± 13 ab	21	21 ± 15 a	31
100	37 ± 16 b	22	33 ± 18 b	38	39 ± 14 b	29

[a] Only direct flights between cage and source were considered.
[b] Flight durations followed by different letters within the same column are significantly different ($P = 0.05$; Kruskal-Wallis test).

14:Ac and the Δ11-components vary widely between individual females (Witzgall and Frérot, 1989). This is apparently mirrored in the male response to such blends. However, it remains to be determined whether all males respond to a wide range of Δ11/Z9 ratios or if the male population is heterogenous with respect to blend perception.

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ROLES OF MINOR COMPONENTS IN PHEROMONE-MEDIATED BEHAVIOR OF WESTERN SPRUCE BUDWORM MALE MOTHS

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Abstract—The behavior of male western spruce budworm moths, *Choristoneura occidentalis* Freeman, was observed in a flight tunnel in response to virgin females and synthetic sex pheromone components, alone and in blends. Pheromone blends were also compared in the field using sticky trap bioassays. Pheromones were incorporated into small rods of polyvinyl chloride. The blend of 92:8 (*E*/*Z*)-11-tetradecenal–89:11 (*E*/*Z*)-11-tetradecenyl acetate–85:15 (*E*/*Z*)-11-tetradecenol (Ald:Ac:OH) that approximated that released from a virgin female moth elicited levels of response similar to those elicited by the female. This blend induced a significantly greater percentage of moths to fly upwind and land at the lure than did the Ald lure. In contrast to the flight-tunnel bioassays, the numbers of moths caught in Ald-baited sticky traps in the field were not significantly increased by the addition of Ac and OH lures. The net upwind groundspeed of flight in response to the 0.05% Ald lure was lower than that in response to the virgin females and was significantly increased by the addition of Ac + OH lures in two of three bioassays. The flight-tunnel bioassays support the hypothesis that the natural blend of major (Ald) and minor (Ac + OH) components stimulates the precopulatory behavior of western spruce budworm male moths at long range (>1 m downwind) as well as at close range.

Key Words—*Choristoneura occidentalis*, Lepidoptera, Tortricidae, sex pheromones, behavior, flight tunnel, minor components, electroantennograms.

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INTRODUCTION

Roelofs and Cardé (1977) proposed that multicomponent pheromones consisted of primary components, responsible for eliciting long-range (>1 m) upwind orientation, and secondary components, which in combination with primary components elicit close-range behaviors such as landing and copulation. This model was supported by studies of the Oriental fruit moth (Cardé et al., 1975), the redbanded leafroller (Baker et al., 1976), the cabbage looper (Linn and Gaston, 1981a,b), and the pine beauty moth (Bradshaw et al., 1983). However, subsequent studies (Baker and Cardé, 1979; Baker and Roelofs, 1981; Linn et al., 1986) have suggested that the entire blend of components acts as a unit to elicit both long-range (early) and close-range (late) behaviors.

The sex pheromone of the western spruce budworm, *Choristoneura occidentalis* Freeman, is a blend of 92:8 (*E*/*Z*)-11-tetradecenal (Ald); 89:11 (*E*/*Z*)-11-tetradecenyl acetate (Ac); and 85:15 (*E*/*Z*)-11-tetradecenol (OH), in a relative ratio of about 10:3:6, respectively, plus tetradecanal (14:Ald) and tetradecanyl acetate (14:Ac) at about 1–2% of the Ald (Silk et al., 1982; Cory et al., 1982). Weatherston et al. (1971) demonstrated that male western spruce budworm moths were attracted to (*E*)-11-tetradecenal in the field. Cory et al. (1982) found that catches in sticky traps were increased by adding 3–8% of the *Z* isomer to (*E*)-11-tetradecenal but were not affected by adding Ac or OH, either alone or together. Similarly, in sympatric populations of *C. occidentalis* and *C. retiniana* (Walsingham), Liebhold and Volney (1985) found the percentage of *C. occidentalis* caught in Ald-baited sticky traps was not affected by the addition of low concentrations of Ac and OH. Cory et al. (1982) suggested that Ac and OH were unnecessary for long-range attraction of males but could possibly have close-range behavioral roles not detectable by trapping bioassays. However, in flight-tunnel experiments using release rates approximating those of a virgin female, Alford and Silk (1984) found that the percentage of moths flying upwind to an Ald lure was significantly increased by adding Ac, OH, or both; neither Ac nor OH were attractive by themselves. The blend of Ald–Ac–OH (10:10:50 ng on filter paper) induced as much upwind flight as a virgin female but elicited significantly fewer landings and copulatory attempts at the lure (Alford and Silk, 1984). It was apparent that Ac and OH enhanced the orientation response of male western spruce budworm to Ald but no blend induced so complete a behavioral sequence as a virgin female. Alford and Silk (1984) suggested that their Ald–Ac–OH blend ratios were different from those emitted from a virgin female because of unequal release rates of the components from filter paper.

Using bioassays in the flight tunnel and the field, we tested the following hypotheses: (1) the orientation and precopulatory behavior of the male western

spruce budworm moth in response to the major sex pheromone component, Ald, is affected by the addition of the minor compnoents, Ac or OH; and (2) the minor components do not stimulate merely close-range behavior but, in combination with the major component, Ald, they stimulate the male moth's behavior at long range (>1 m) as well.

METHODS AND MATERIALS

Insects. The moths were reared from crosses between female budworm collected as pupae in the field, and male budworm from a nondiapausing, laboratory strain obtained from the Forest Pest Management Institute (FPMI), Forestry Canada, in Sault Ste. Marie, Ontario. Larvae were reared following the methods outlined by Robertson (1979) and Sweeney and McLean (1987).

Pheromones. The pheromones for laboratory and field bioassays were synthesized by L. Weiler (Department of Chemistry, University of British Columbia, Vancouver). Each component isomer was determined to be greater than 99% pure by gas–liquid chromatography (GLC); the (*E*)-11-tetradecenyl acetate contained 0.2% (*E*)-11-tetradecenal and 0.2% (*E*)-11-tetradecenol. Unless specified otherwise, all pheromone components were blended at the natural isomer ratios, e.g., 92% (*E*)- + 8% (*Z*)-11-tetradecenal, and incorporated into polyvinyl chloride (PVC) rods of 3 mm diam. (Daterman, 1974) at concentrations of 0.005%, 0.05%, and 0.5% (w/w). The rods (lures) were then cut into 5-mm lengths unless otherwise noted. Different pheromone blends were then made by impaling more than one PVC rod on a pin, e.g., a 0.05% Ald rod plus a 0.5% Ac rod. All PVC lures were aged seven days in a fume hood (23–25°C) before use in the flight tunnel or in field tests. They were stored in separate sealed glass vials at -10°C when not in use.

Release rates of 0.05% and 0.5% Ald, 0.5% Ac, and 0.5% OH lures were estimated by capturing volatiles on Porapak-Q. The Ald used in the determination of release rates was from a commercial supplier (Orsynex) and was in a 97:3 *E/Z* isomer ratio; the Ac and OH lures were the same as those used in laboratory and field experiments. For each component concentration, one 10- to 15-cm length of PVC was aged in a fume hood at 25 $\pm$ 1°C for seven days, and was then placed in a glass aeration chamber fed by compressed air (24–26°C; 157 ml/sec), which passed through a water bath, to control its temperature, and activated charcoal. Volatiles were trapped on Porapak-Q for 67 hr. Two columns of Porapak-Q were connected in series for each aeration device. The Porapak-Q was later eluted with four rinses of 5 ml ether and any rinses that contained pheromone were later combined. Trapped volatiles were analyzed by GLC in the manner described for the determinations of pheromone

purity (Sweeney and McLean, 1990). Tetradecane was used as the internal standard. Release rates were calculated per unit surface area of PVC and expressed on a per hour basis for a 5 × 3-mm lure.

Flight-Tunnel Bioassays. The flight tunnel was 3.6 m long and 1.2 m in cross section and was fitted with activated charcoal filters and a dust filter on the upwind screen; it was modified from an earlier version (Angerilli and McLean, 1984). Air was pulled through the tunnel and expelled to the outside of the building. For all experiments, the windspeed was 40 cm/sec, light intensity was 35 lux, and the temperature was between 20 and 25°C.

Behavior of the male moths was observed in response to Ald (0.05%), Ac (0.005%, 0.05%, 0.5%), and OH (0.005%, 0.05%, 0.5%), alone and in blends, in three flight-tunnel bioassays corresponding to the three concentrations of minor components. Each bioassay included: a 0.05% Ald lure; a virgin female treatment that consisted of two virgin female moths, aged 2–6 days, inside a fiberglass screen cage; and a control treatment that consisted of a PVC lure to which only heptane had been added. Male moths were released individually, 2 m downwind from the treatment in the flight tunnel, and their behavior was observed and timed. We noted the percentage of moths that wing-fanned, took flight, locked-on (zigzag flight in the pheromone plume), flew upwind, landed at the lure, and displayed copulatory behavior (for details see Sweeney and McLean, 1990). Treatment order was randomized each day, and five males were flown consecutively to each treatment on each of eight test days per bioassay. For percentage response, each replicate consisted of five males, whereas for temporal response, each responding male was considered a replicate. The moths were flown only once and then discarded. Each bioassay was completed within a period of 8–23 days between October 1986 and June 1987. Percent response data were transformed by arcsin ($\sqrt{y}$) and analyzed initially as a randomized complete block design (blocks = days) by ANOVA and Newman-Keuls multiple range test ($\alpha = 0.05$). If the difference between test days was not significant, the variances were pooled and the experiment analyzed as a one-way ANOVA. The time interval and groundspeed data were transformed by $\log(y + 1)$ and compared between treatments by one-way ANOVA and Newman-Keuls multiple range test ($\alpha = 0.05$).

Field Bioassays. The effect of adding various concentrations of OH, Ac, and Ac + OH to 0.05% Ald lures on the catch of male moths in sticky traps was assessed in three experiments located in light to moderate budworm infestations in the Oregon Jack Creek valley, near Ashcroft, British Columbia, in 1984. The experimental design and methods of trap deployment were as described in Sweeney and McLean (1990). To minimize the effects of trap saturation, the sticky traps were replaced and the treatments rerandomized before 50 moths were caught in the traps whenever possible. The numbers of moths

caught were transformed by $\log(y + 1)$ and analyzed by three-way ANOVA and Newman-Keuls multiple range test ($\alpha = 0.05$).

RESULTS

Pheromone Release Rates. The release rates of 0.5% Ald, 0.5% Ac, and 0.5% OH lures were 64.6 ng/hr, 7.8 ng/hr, and 32.2 ng/hr, respectively. When the lures are combined, this converts to a relative ratio of 10:1.2:5 (Ald–Ac–OH), which is comparable to the ratio of 10:3:6 released by the nondiapausing strain of virgin female western spruce budworm (Silk et al., 1982). Our component ratio is also similar to that found by Daterman et al. (1982) (15:8:10), who measured the weight loss of PVC lures loaded with 2% of each component.

The release rate of 0.05% Ald was 4.4 ng/hr. This is comparable to release rates of 5.9 ng/female night equivalent (FNE) (diapausing strain) and 13.5 ng/FNE (nondiapausing strain) (Silk et al., 1982) and 2–5 ng/hr (Cory et al., 1982) found for virgin female western spruce budworm. Consequently, the 0.05% Ald lure was used in blend tests that assayed the effect of adding minor components.

In addition to releasing 7.8 ng/hr of Ac, the 0.5% Ac lure released contaminants of Ald and OH at the respective rates of 2.0 ng/hr and 10.1 ng/hr. This was surprising because the neat (*E*)-11-tetradecenyl acetate contained only 0.2% of the aldehyde and 0.2% of the alcohol. It is possible that the pheromones had deteriorated or had reacted with chemicals in the PVC during formulation of the lures. Although we did not collect volatiles from the 92:8 *E*/*Z* 11–14:Ald lures that we used in the flight tunnel and field experiments, we found that a 0.5% lure made from a 97:3 *E*/*Z*11–14:Ald formulation (purchased from a commercial supplier) released 20.7 ng/hr of OH in addition to 64.6 ng/hr Ald. We assumed that the commercial formulation adequately represented the release rate (and possibly the deterioration) of the Ald formulation used in our behavioral work, because both formulations were greater than 99% pure according to the GLC analysis.

Because of the Ald and OH contaminants measured in the Ac lure volatiles, we could not determine the effects of a binary mixture of Ald and Ac on the male moths behavior. However, a combination of Ald and Ac lures that approximated the blend released by a virgin female was made up based on the estimated release rates of each component. The 0.05% Ald lure released aldehyde at about 6.8% of the rate of the 0.5% Ald lure. We assumed a similar relationship for Ac and OH and calculated that the 0.05% Ac lure and the 0.05% OH lure would release about 0.53 ng/hr acetate and 2.19 ng/hr alcohol, respectively. We also assumed that release rates were uniform per unit surface area of PVC and calculated that a 10:2.8:6.6 ratio of Ald–Ac–OH would be

approximated by a combination of 0.05% Ald (5 × 3 mm) plus two 0.05% Ac lures, each measuring 6.5 × 3 mm. This combination was tested in the flight tunnel along with other lure blends.

Percentage Response in Flight-Tunnel Bioassays. The percentage of moths wing-fanning, taking-off, and flying upwind to land at the lure was significantly greater in response to any combination that contained the Ald lure than to either Ac or OH lures or the blank PVC control. Significantly greater percentages of moths took flight in response to the 0.5% Ac (55%), 0.5% OH (40%), and the 0.05% OH lures (58%) than to the blank PVC control (20–30%). Otherwise, the Ac and OH lures, by themselves, had no significant effects on the moth's behavior despite the Ald contamination measured in the Ac lure volatiles.

The percentage responses to the virgin female moths, Ald lures, or blends of Ald, Ac, and OH are presented in Figure 1A–C. In two of the three bioassays, the percentages of moths wing-fanning, flying upwind, and reaching a virgin female lure were significantly greater than those responding to a 0.05% Ald lure (Figure 1B and C).

Response to the ternary combination of 0.05% Ald + 0.5% Ac + 0.5% OH lures did not differ significantly from that to the 0.05% Ald lure (Figure 1A). However, the combination of two 0.05% Ac lures (6.5 × 3 mm PVC rods) and the 0.05% Ald lure (releasing Ald–Ac–OH at a 10:2.8:6.6 ratio, approximating a virgin female), elicited similar percentage responses as the virgin females and significantly greater percentages of upwind flight and landing at the lure than did the 0.05% Ald lure alone (Figure 1B).

Furthermore, the blends made up of a 0.05% Ald lure plus either the 0.05% Ac + 0.05% OH lures or the 0.005% Ac + 0.005% OH lures, elicited significantly greater percentage landing at the lure and copulatory attempts than did the 0.05% Ald lure. Percentage response to these blends was not significantly different from that elicited by the virgin females except that significantly fewer moths wing-fanned in response to the lure combination of 0.05% Ald + 0.005% Ac + 0.005% OH (Figures 1B and C).

Temporal Responses in Flight-Tunnel Bioassays. Moths took significantly longer to begin wing-fanning in response to 0.05% Ald + 0.05% Ac (mean = 19 sec) than to the virgin females (mean = 6 sec). This was surprising because this combination approximated the blend and release rate of a virgin female and had elicited similar percentage responses. For all other blends that contained 0.05% Ald, the mean elapsed time between release of the moth on the platform and the onset of wing-fanning ranged from 2 to 8 sec and was not significantly different from that in response to the virgin females. The mean elapsed time between release of the moth on the platform and the initiation of flight was significantly longer in response to 0.5% Ac (51 sec) and 0.5% OH (43 sec) than to 0.05% Ald (21 sec), blends of 0.5% Ac, 0.5% OH, and 0.05% Ald (13–18 sec), or the virgin females (21 sec).

The mean net groundspeed of male moths approaching a 0.05% Ald lure

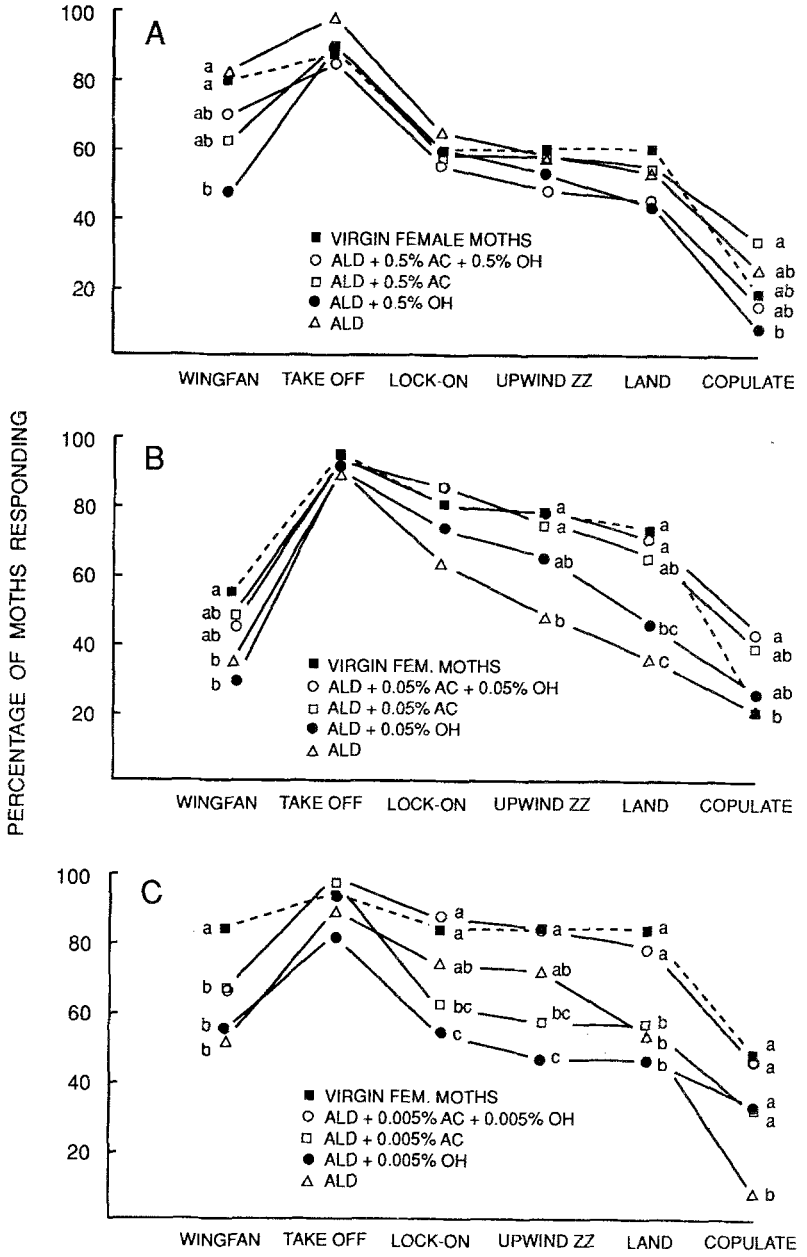


FIG. 1. Percentage response of western spruce budworm male moths in a flight tunnel to lure combinations of 0.05% Ald and varying concentrations of Ac (also emitting Ald and OH contaminants) and OH. The lure concentrations of Ac and OH were: (A) 0.5% (in 5 × 3-mm PVC lures); (B) 0.05% (two 6.5 × 3-mm PVC lures of Ac and one 7 × 3-mm PVC lure of OH; (C) 0.005% (in 5 × 3-mm PVC lures). For each bioassay, the means within a behavioral variable with a different letter are significantly different [ANOVA and Newman-Keuls, $\alpha = 0.05$, data transformed by arcsin $(\sqrt{y})$]. Eight replicates of five moths each were tested for response to each pheromone treatment in each bioassay.

from 0.5 m downwind was significantly increased by the addition of either Ac or Ac + OH, at concentrations of 0.5% and 0.05%; groundspeeds in response to these blends were not significantly different from those in response to the virgin females (Figure 2A and B). Most blends did not affect the net groundspeed of moths at distances greater than 0.5 m downwind. However, the blend of the 0.05% Ald + 0.05% Ac lures stimulated significantly faster upwind net groundspeeds of flight at distances downwind of 2 m to 1 m (mean = 24 cm/sec) and 1 m to 0.5 m (mean = 18 cm/sec) than did the 0.05% Ald lure (mean = 12 cm/sec and 10 cm/sec, respectively). The net groundspeed of flight in response to 0.05% Ald was not significantly affected by the addition of OH at any concentration (Figure 2A–C).

Field Bioassays. Moth catch in traps baited with a 0.05% Ald lure was not significantly affected by adding any concentration of OH (Figure 3A) but was significantly reduced by adding 0.5% Ac (Figure 3B). The ternary blend of 0.05% Ald + 0.005% Ac + 0.005% OH caught 49% more moths than did the 0.05% Ald lure alone, but the difference was not significant (Figure 3C). Traps baited with virgin females caught fewer moths than those baited with 0.05% Ald or combinations of Ald with minor components in all three field bioassays.

DISCUSSION

The results from the flight-tunnel bioassays support the hypothesis that the precopulatory behavior of western spruce budworm male moths is stimulated by a blend of major and minor pheromone components and not just the major component, Ald. The combination of 0.05% Ald + 0.05% Ac + 0.05% OH lures, and the combination of 0.05% Ald + two 0.05% Ac lures, that according to GC analysis approximated the ternary blend from a virgin female moth, elicited percentage responses similar to those elicited by the female and significantly greater percentage upwind flight and landing at the lure than did the 0.05% Ald lure (Figure 1B).

Further evidence that male moths were stimulated by a blend of major and minor components rather than the Ald alone was provided by two of the three bioassays in which the virgin females elicited significantly greater percentages of wing-fanning, upwind flight, and landing at the lure than did the 0.05% Ald lure (Figure 1B and C). The lack of a significant difference between the virgin female and the 0.05% Ald lure in one of the bioassays (Figure 1A) may have been due to variability in the frequency of calling or in the pheromone release rate of individual females. Although the female moths were checked periodically for calling posture during the course of a bioassay, it was difficult to ensure that they were calling at all times. Considerable variability in pheromone release rates and component ratios has been observed in virgin females of *C. fumiferana* (Morse et al., 1982), *C. occidentalis* (Silk et al., 1982), and other moth species (Barrer et al., 1987; Haynes et al., 1984; Pope et al., 1982).

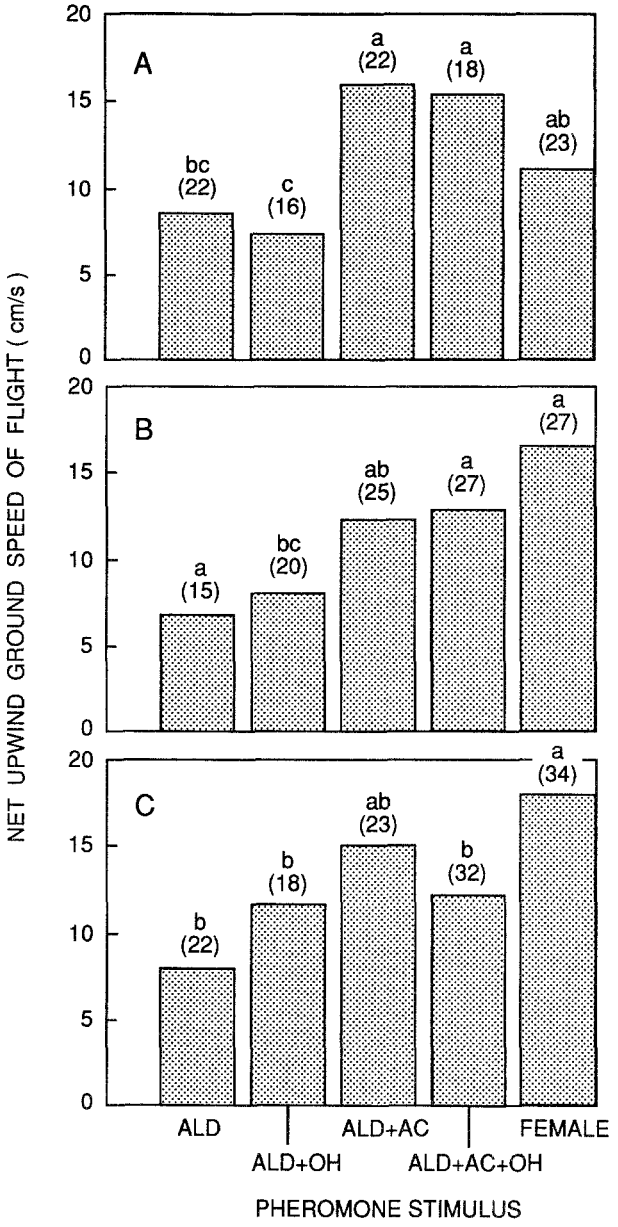
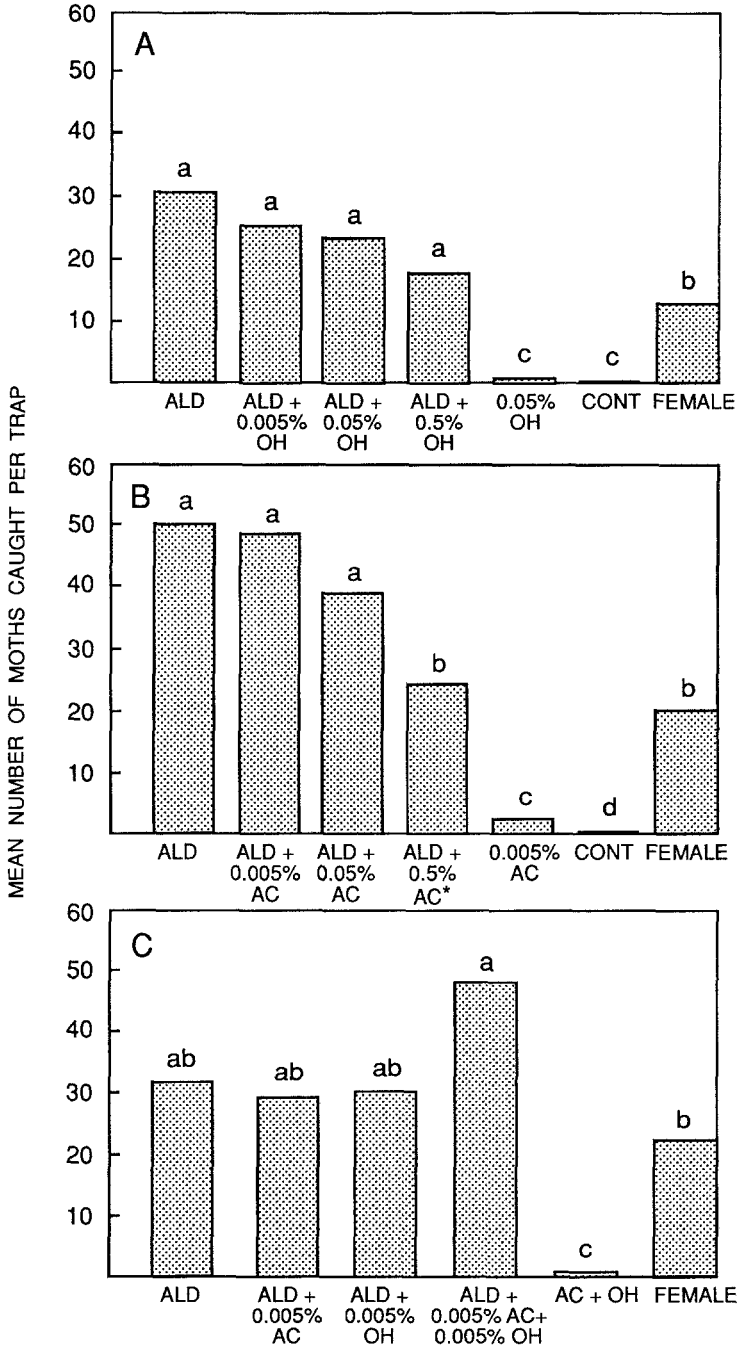


FIG. 2. The mean net upwind groundspeed of flight and western spruce budworm male moths, from 0.5 m downwind until contact with the lure, in response to synthetic pheromone blends or two virgin female moths. A 0.05% Ald lure was combined with Ac (also emitting Ald and OH contaminants) and OH lures at concentrations of: (A) 0.5%; (B) 0.05%; and (C) 0.005% in PVC. Within each experiment, means having different letters are significantly different by ANOVA and Newman-Keuls ($\alpha = 0.05$). The data were transformed by $\log(y + 1)$. The number of moths responding to each treatment is indicated within each bar.


MEAN NUMBER OF MOTHS CAUGHT PER TRAP
A
60
50
40
30
20
10
a
a
a
a
c
c
b
ALD
ALD + 0.005% OH
ALD + 0.05% OH
ALD + 0.5% OH
0.05% OH
CONT
FEMALE
B
a
a
a
b
c
d
b
ALD
ALD + 0.005% AC
ALD + 0.05% AC
ALD + 0.5% AC*
0.005% AC
CONT
FEMALE
C
ab
ab
ab
a
c
b
ALD
ALD + 0.005% AC
ALD + 0.005% OH
ALD + 0.005% AC+ 0.005% OH
AC + OH
FEMALE

Ternary blends of major and minor components also increased the speed of net upwind progress of the male moths in the flight tunnel relative to a 0.05% Ald lure (Figure 2A and B). Sanders et al. (1981) found that the net upwind groundspeed of *C. fumiferana* male moths was significantly faster in response to two virgin females than to PVC lures releasing 96:4 (*E*/*Z*)-11-tetradecenal at about the same rate and suggested that additional minor components in the female effluvia were possibly responsible. Our data support a similar hypothesis for the western spruce budworm.

The role of the minor components in the stimulation of copulatory behavior was not clear. The ternary blends elicited significantly more attempted copulation than did the 0.05% Ald lure in two bioassays (Figure 1B and C), but the percentage response to all treatments was fairly low. It is likely that visual and tactile stimuli also affect the copulatory behavior of the western spruce budworm. Grant (1987) found that *C. fumiferana* male moths only attempted copulation in the presence of synthetic sex pheromone when appropriate tactile stimuli such as moth scales or rubber septa were also present. The copulatory attempts that we observed on the outside of the fiberglass cages may have been influenced by wing scales left on the cages by previous moths or by the surface of the cages themselves.

In contrast to the flight-tunnel bioassays, the field-trapping bioassays showed very few significant differences between blends of Ald, Ac, and OH, and the Ald alone. Although we made an effort to avoid trap saturation, we recorded catches of 50 moths per trap for some blends, and this may have obscured differences in their effects. Further field bioassays of these blends should be done using nonsticky traps.

Our findings that traps baited with virgin females caught less than those baited with 0.05% Ald, or blends containing Ald, also conflict with the flight-tunnel observations. Others have shown that catches of male moths in triangular sticky traps baited with synthetic lures, at release rates about that of a virgin female, are often greater than the catches in traps baited with virgin female *Choristoneura orae* (Gray et al., 1984a), *C. retiniana* (Daterman et al., 1984), *Rhyacionia buoliana* (Gray et al., 1984b) and *C. occidentalis* (Sweeney and McLean, 1990). Conditions inside the sticky traps may have affected the results.

FIG. 3. Mean numbers of western spruce budworm moths caught in triangular sticky traps baited with synthetic pheromone (in 5 × 3-mm PVC lures) or a virgin female moth. A 0.05% Ald lure combined with: (A) OH lures at 0.5%, 0.05%, and 0.005% concentrations ($N = 8$); (B) AC lures at 0.5%, 0.05%, and 0.005% concentrations ($N = 8$) (also emitting Ald and OH contaminants); and (C) Ac and OH lures at 0.005% concentration ($N = 7$). Means within bioassays with different letters are significantly different (ANOVA and Newman-Keuls on data transformed by the $\log(y + 1)$; $\alpha = 0.05$).

We sometimes found virgin females caught in adhesive that had oozed through the fiberglass cage from the walls of the sticky trap. Temperatures in the field may have resulted in higher net release rates from the synthetic lures than those measured under controlled laboratory conditions. The mean peak ambient temperature during the bioassays was 30°C and the temperature inside traps exposed to the sun was probably higher. High temperatures may also have adversely affected the caged females.

The flight-tunnel results support the hypothesis (Linn et al., 1986; 1987) that the blend of major and minor pheromone components stimulates upwind flight and other behaviors of the male moth at long range (>1 m downwind) as well as at close range. Significant differences were noted in the percentage of moths that locked-on 2 m downwind from different blends (Figure 1C). At distances greater than 1 m from the lure, the synthetic blend of Ald, Ac, and OH that approximated a virgin female moth stimulated a significantly greater percentage of moths to fly upwind than did the 0.05% Ald lure; this lure also induced moths to fly toward the lure with a greater net upwind groundspeed. The effect of pheromone blend on longer range (>20 m) responses of western spruce budworm moths should be tested using the sort of field bioassays described by Linn et al. (1987).

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EFFECT OF SEX-PHEROMONE CONCENTRATION ON BEHAVIOR OF THREE STRAINS OF WESTERN SPRUCE BUDWORM MALE MOTHS

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Abstract—The responses of male western spruce budworm moths, *Choristoneura occidentalis* Freeman, to a range of concentrations of the major sex pheromone, 92:8 (*E*/*Z*)-11-tetradecenal (Ald), in polyvinyl chloride lures, were observed using the electroantennogram technique, a flight tunnel, and field-trapping bioassays. The responses to virgin female moths were also observed in the flight tunnel and field bioassays. The moths were from three strains: a nondiapausing laboratory colony; field-collected wild budworm; and laboratory–wild crosses. The mean peak amplitude of antennal response and the time required for the electroantennogram signal to return to the baseline after stimulation (lag) increased with Ald concentration in both laboratory and wild moths. However, at Ald concentrations of 0.005% and greater, the lag period of the wild male's antennae was significantly shorter than that of the laboratory male's. The mean number of moths caught in the field in delta sticky traps increased with Ald concentration, but the number of moths caught per trap was not significantly different between concentrations of 0.005% and 0.5%. The threshold concentration required to elicit upwind flight in the flight tunnel was between 0.0005 and 0.005% Ald; peak response occurred to 0.05% Ald but was not significantly different from that to 0.005% or 0.5% Ald. Moths from all three populations significantly reduced their net upwind groundspeed as they approached the pheromone lure. When pheromone concentration was increased, the net upwind groundspeed of laboratory and lab–wild moths, but not wild moths, was significantly reduced between 2 m and 1 m downwind from the pheromone lure. The three populations of moths differed significantly in the percentage of wing-fanning and copulatory attempts, and in the net upwind groundspeed of flight from 2 m to 1 m downwind from the lure.

Key Words—*Choristoneura occidentalis*, Lepidoptera, Tortricidae, sex pheromone concentration, behavior, flight tunnel, population differences.

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INTRODUCTION

Pheromone concentration has been shown to influence the probability of upwind anemotactic flight (Roelofs, 1978) and the rate of upwind progress (Kuenen and Baker, 1982a,b) of male moths towards a pheromone source. Roelofs (1978) hypothesized that stimulation of upwind flight to a given pheromone blend occurred at concentrations that were above an activation threshold but below an arrestment threshold. This hypothesis has been supported by several studies of pheromone-mediated behavior in moths (e.g., Baker and Roelofs, 1981; Baker et al., 1981; Bellas and Bartell, 1983; Mankin et al., 1980).

An inverse relationship between net upwind groundspeed and pheromone concentration has been found in the gypsy moth, *Lymantria dispar* L. (Cardé and Hagaman, 1979), the Oriental fruit moth (Kuenen and Baker, 1982a), and the spruce budworm, *Choristoneura fumiferana* (Clem.) (Sanders et al., 1981). In contrast, Linn and Gaston (1981) observed no change in upwind flight speed of adult male cabbage loopers, *Trichoplusia ni* (Hübner), associated with pheromone concentration.

Using the electroantennogram technique, a laboratory flight tunnel, and field bioassays, we observed the responses of western spruce budworm male moths, *Choristoneura occidentalis* Freeman, to a range of concentrations of its major sex pheromone component, 92 : 8 (*E*/*Z*)-11-tetradecenal (Ald) (Silk et al., 1982; Cory et al., 1982). The moths were from three strains: a laboratory colony, field collections of wild moths, and laboratory–wild crosses. We tested the following hypotheses: (1) the stimulation of flight upwind to a pheromone lure requires concentrations that are above an activation threshold but below an arrestment threshold (Roelofs, 1978); (2) the net upwind groundspeed of the male moth's flight is inversely related to pheromone concentration; and (3) responses to pheromone differ among moth strains.

METHODS AND MATERIALS

Insects. The laboratory colony was started from a strain of nondiapausing western spruce budworm maintained at the Forest Pest Management Institute (FPMI), Forestry Canada, in Sault Ste. Marie, Ontario. The FPMI colony originated from a cross between males from the USDA Berkeley strain (generation 90) and females from a colony maintained at the Pacific Northwest Forest Experimental Station in Corvallis, Oregon, and had been bred in the laboratory for about 30 generations. Wild larvae were collected as fifth and sixth instars from a moderate infestation in the Oregon Jack Creek valley near Ashcroft, British Columbia (BC) and were reared on current year's Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, foliage at 28 ± 2°C, ambient humidity, and a 16 : 8 light–dark photoperiod with scotophase starting at 1500 hr (PST).

Laboratory and lab–wild larvae were fed an artificial diet (Robertson, 1985) that contained Benomyl at 145 ppm to control the level of *Nosema* sp. and were reared following the methods outlined by Robertson (1979) and Sweeney and McLean (1987). The pupae were sexed and males to be used in behavioral tests were placed in individual 180-ml plastic cups and stored at 28 ± 2°C, ambient humidity, and a 16:8 light–dark photoperiod with scotophase starting at 1500 hr (PST). Adult emergence was checked daily. Adults were flown in the flight tunnel between two and six days from eclosion.

Pheromones. The pheromones were synthesized by Dr. L. Weiler (Department of Chemistry, University of British Columbia, Vancouver). Both the *E* and *Z* isomers of Ald were found to be 99% pure as measured on a Hewlett Packard 5890A gas chromatograph (GC) equipped with an HP 3390A integrator and run on a splitless mode column (DB1701, 30 m × 1.0μ).

The isomers were blended in the natural ratio, 92% (*E*)- + 8% (*Z*)-11-tetradecenal, and incorporated into polyvinyl chloride (PVC) rods of 3 mm diam (Daterman, 1974) at concentrations of 0.00005%, 0.0005%, 0.005%, 0.05%, and 0.5% (w/w). A control lure was made up by adding heptane only to PVC. The rods (lures) were then cut into 5-mm lengths. All PVC lures were aged seven days in a fume hood (23–25°C) before use in the electroantennogram bioassays, the flight tunnel, or field tests. They were stored at −10°C when not in use. Release rates from 0.5% and 0.05% PVC lures (5 × 3-mm rods) of commercial 97:3 (*E*/*Z*)-11-tetradecenal (Orsynex) were 64.6 ng/hr and 4.4 ng/hr, respectively (Sweeney et al., 1990). The release rate of 4.4 ng/hr for 0.05% Ald is comparable with release rates of Ald measured from western spruce budworm virgin females by Silk et al. (1982) and Cory et al. (1982).

Electroantennograms (EAGs). Antennal responses of laboratory and wild male moths to pheromone were recorded using materials and methods similar to those described by Roelofs (1977). Modifications included a Faraday cage to reduce electrical noise and a power supply that automatically reset the baseline after each EAG recording. The EAG power supply was built using a modified design of Perez and Rozas (1984). Two variables were recorded from the EAG: the peak amplitude of depolarization and the time required for the signal to return to the baseline (lag). For each replicate, a moth antenna was tested for response to Ald at each of the above concentrations in PVC. The PVC lure was wedged crosswise into the large end of a Pasteur pipet that was then fitted onto a large syringe. Air was puffed in 5-cc quantities through the pipet and into a stream of medical air directed at the antenna. Different pipets were used for each pheromone concentration and treatments were tested in order of increasing concentration. A control stimulus (PVC without pheromone) was puffed three times over each antenna before and after each pheromone concentration was tested. The mean amplitude and lag before return to the baseline in response to the control puffs was subtracted from the mean response to three puffs of pheromone. Simple linear regressions of amplitude vs. Ald concentra-

tion [log ($x * 10^4$)] were calculated for each budworm population and were compared for slope and intercept ($P \leq 0.05$; Zar, 1974). The laboratory and wild males were tested for differences in response to each pheromone concentration using the Mann-Whitney U test ($P \leq 0.05$).

Flight-Tunnel Bioassays. A suction-type flight tunnel (Angerilli and McLean, 1984) was used with the following modifications: the tunnel was shortened to 3.6 m, the baffles were removed from the intake end, and charcoal filters plus a dust filter were fitted to the upwind screen. Activated charcoal was held inside a grid consisting of four plastic filters, each measuring 61 × 56 × 2.5 cm. The windspeed in the tunnel was 40 cm/sec and the temperature was between 20 and 25°C for all experiments. Light intensity was 35 lux.

The response of the male moths was observed to the following: the five concentrations of Ald in PVC; two virgin female moths (aged two to six days); and a control PVC lure. Each treatment was held inside a fiberglass screen cage that was suspended by a paperclip from an aluminum rod held 40 cm above the center of the tunnel floor. Male moths were flown from 2 hr before to 3 hr after the start of scotophase (1300–1700 hr PST) in order to coincide with the male's pheromone response period (Shepherd, 1979; Liebhold and Volney, 1984). The males were acclimatized to room conditions for at least 30 min and then released individually from a plastic cup onto a glass platform located 40 cm above the flight tunnel floor and 2 m downwind from the lure. Each male was given 3 min in which to respond and was observed for the following behavior: wing-fanning, taking off, zigzag flight in the pheromone plume with no net upwind progress (locking on), flying a zigzag pattern upwind towards the lure (upwind flight), touching and landing on the cage containing the pheromone, and displaying postflight precopulatory behavior (wing-fanning while curling the abdomen to the side and displaying hair pencils). A male was considered to have landed on the cage if it remained there for longer than 5 sec. All moths that failed to take off or flew to the floor were scored as nonfliers and discarded if they failed to fly after three launches above the flight tunnel floor.

The mean net groundspeed of upwind flight was calculated in three sections of the flight tunnel (2–1 m, 1–0.5 m, and 0.5–0 m from the lure) and averaged over the entire flight tunnel from the start of upwind flight. Also calculated were: the interval between release of the moth and the onset of wing-fanning and take-off; between taking off and subsequently locking on; between locking on and subsequently flying upwind or flying out of the plume; and between first touching the cage and then landing on it.

On each test day, the order of pheromone treatments was randomized, and five male moths were tested consecutively for response to each treatment. The pheromone treatments were replicated in a randomized complete block design (blocks = days). For the percentage response data, a replicate consisted of five males; for the temporal variables, each responding male was used as a replicate. Males were flown only once per day, but some were used once again on a subsequent day.

The percentage response data were transformed by arcsin ($\sqrt{y}$) and the time interval and flight speed data were transformed by log ($y + 1$) to reduce heterogeneity of variance. There were minimal differences between test days so the variance resulting from blocks was pooled in the error term. The data for each behavioral variable were analyzed by two-way ANOVA (SAS Institute, General Linear Model procedure); if moth populations differed significantly or there was a significant interaction between population and pheromone treatment, then the data for that variable were analyzed separately for each population. Means were compared using the Newman-Keuls test ($\alpha = 0.05$). For each moth population, the net groundspeeds of flight in the three flight tunnel sections were compared using paired t tests ($\alpha = 0.05$).

Field Bioassay. Trapping took place in a light to moderate budworm infestation in the Oregon Jack Creek valley, near Ashcroft, BC. Sticky traps, made from 2-liter milk cartons, folded into triangles (Cory et al., 1982), were baited with 0.0005%, 0.005, 0.05, 0.5% Ald or a virgin female moth. The female was held inside a fiberglass screen cage pinned to the inside roof of the trap. Females were attractive between two and six days from eclosion. Traps were hung 1.5–2 m above the ground on live branches and were aligned with their long axis parallel to the prevailing evening winds. The traps were spaced 25 m apart in a single line perpendicular to the prevailing winds in order to minimize trap and plume interference. The position of each treatment in the line was rerandomized according to a Latin-square design (rows = position, columns = time interval) so that each treatment appeared once in each position. To minimize the effects of saturation, the traps were replaced and the treatments rerandomized, whenever possible, before 50 males were caught in the traps. Data were transformed by log ($y + 1$) and analyzed by three-way ANOVA and Newman-Keuls ($\alpha = 0.05$). The variances of transformed data were homogeneous or close to homogeneity in all cases (Box test; $\alpha = 0.05$) (Box, 1949).

RESULTS

Electroantennograms. The mean peak amplitude of antennal response increased with increasing concentrations of Ald (Figure 1A). The regressions of amplitude vs. concentration were significant ($P \leq 0.001$) for both laboratory ($r^2 = 0.77$) and wild moths ($r^2 = 0.73$), and they were not significantly different from each other ($P \leq 0.05$). The recovery period, or lag, also increased with increasing pheromone concentrations (Figure 1B), but was significantly longer in laboratory males than in wild males.

Percentage Response in Flight Tunnel. The percentages of male moths that took flight, locked on, and flew upwind to land at the various lures were not significantly different among the laboratory, wild, and lab–wild moths. The pooled data are plotted in Figure 2. Significant locking on, upwind flight, and landing at the lure occurred above concentrations of 0.0005% Ald but, except

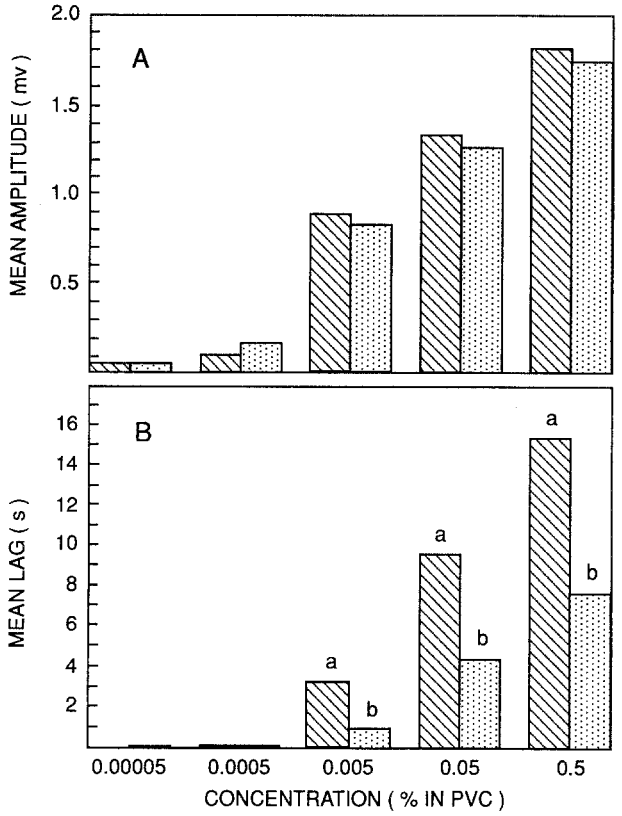

FIG. 1. Mean antennal responses of laboratory reared ▨ (N = 19) and wild ⊡ (N = 20) western spruce budworm male moths to a range of concentrations of 92:8 (E/Z)-11-tetradecenal (Ald) as measured by the electroantennogram technique. Each moth antenna was tested with all concentrations of pheromone. (A) Mean peak amplitude. (B) Time for the signal to return to the baseline (lag). Within concentrations of Ald, a different letter denotes a significant difference in mean response between populations (Mann-Whitney U test, P = 0.05).

for percentage locking on, no significant differences were observed between responses to 0.005%, 0.05%, and 0.5% Ald. The virgin females elicited significantly greater percentages of locking on, upwind flight, and landing at the lure than any Ald concentration except 0.05% (Figure 2).

There were significant differences among the moth populations in the percentage of moths wing-fanning (two-way ANOVA and Newman-Keuls, α = 0.05). In the laboratory and lab–wild moths, pheromone concentrations at or above 0.005% elicited significantly greater percentages of wing-fanning than did the control lure (Figure 3A and C). However, in the wild moths, only the

0.5% Ald lure and the virgin females elicited significantly greater percentages of wing-fanning than the control lure (Figure 3B).

The strains also differed in the percentage of moths that attempted copulation; laboratory males attempted copulation significantly more than wild males (two-way ANOVA and Newman-keuls, $\alpha = 0.05$). However, for all three moth strains, the virgin females elicited the highest percentage of copulatory attempts, and none of the Ald concentrations tested elicited a significantly greater percentage of copulatory attempts than the control lure (Figure 4A–C).

Temporal Response in Flight Tunnel. Male moths began wing-fanning significantly sooner in response to either the virgin females or Ald concentrations at or above 0.05% than to the lower Ald concentrations tested (Table 1). For the wild and lab–wild moths, but not the laboratory moths, the delay between release of the moth and take off was significantly shorter in response to the virgin females than to the blank control (Table 2).

With one exception (0.005% Ald, 1 m to 0.5 m), the net upwind speed of

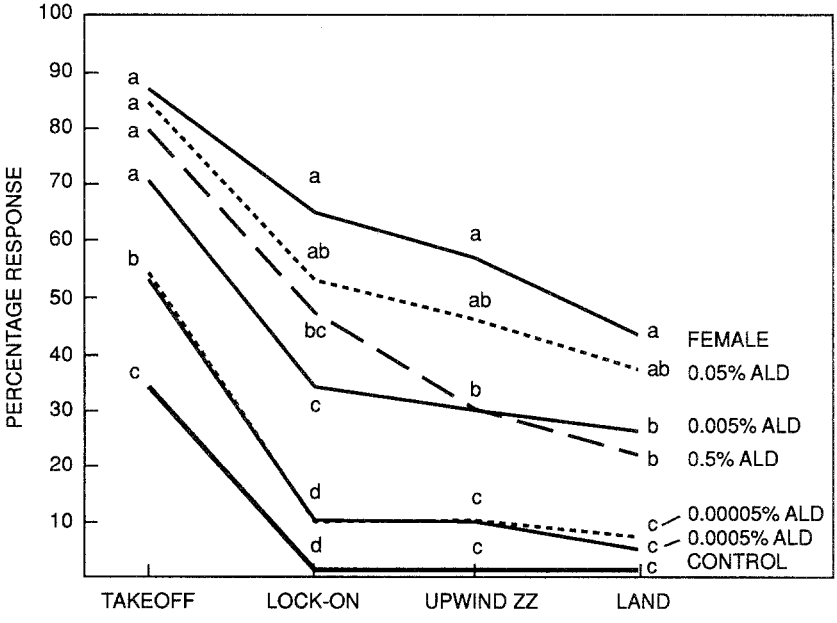

FIG. 2. The percentage of western spruce budworm male moths responding to two virgin female moths and a range of concentrations of 92:8 (*E*/*Z*)-11-tetradecenal (Ald) in a flight tunnel. Data were pooled from a laboratory population, a wild population, and lab–wild crosses. Twenty-three replicates of five moths per replicate were tested for response to each pheromone lure. Means within behavioral variable that are accompanied by a different letter are significantly different (ANOVA and Newman-Keuls test on data transformed by arcsin ($\sqrt{y}$); $\alpha = 0.05$).

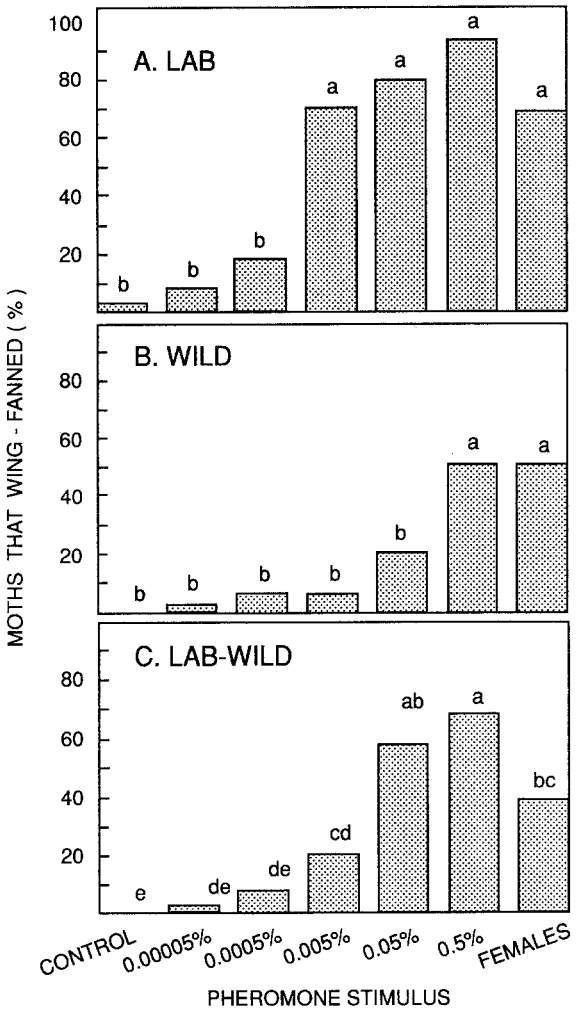

FIG. 3. The percentage of western spruce budworm male moths wing-fanning in response to two virgin female moths and a range of concentrations of 92 : 8 (*E*/Z)-11-tetradecenal in a flight tunnel. (A) Laboratory population ($N = 8$); (B) wild population ($N = 7$); (C) laboratory–wild crosses ($N = 8$). Each replicate consisted of five moths (i.e., when $N = 8$, 40 moths were tested to each treatment). Within each population, means accompanied by a different letter are significantly different (ANOVA and Newman-Keuls test on data transformed by arcsin ($\sqrt{y}$); $\alpha = 0.05$).

flight was significantly faster in response to virgin females than to synthetic Ald from distances of 1 m to 0.5 m downwind, from 0.5 m to the lure, and from the start of upwind flight until reaching the lure (Table 1). At these intervals, there was no trend of decreased flight speed associated with increased phero-

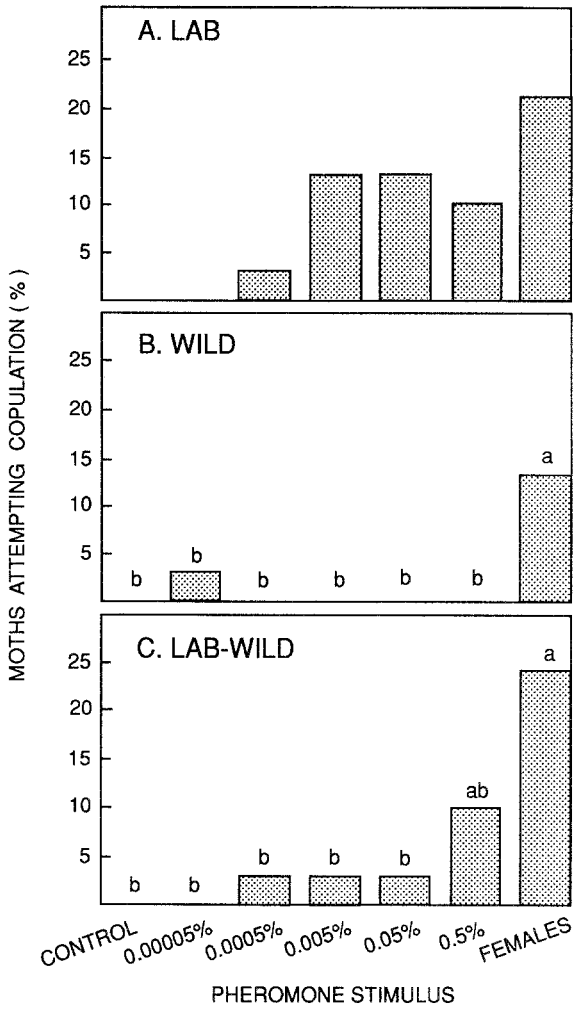

FIG. 4. The percentage of western spruce budworm male moths displaying postflight copulatory behavior in response to two virgin female moths and a range of concentrations of 92:8 (*E*/*Z*)-11-tetradecenal in a flight tunnel. (A) Laboratory population (N = 8); (B) wild population (N = 7); (C) laboratory–wild crosses (N = 8). Each replicate consisted of five moths (i.e., when N = 8, 40 moths were tested to each treatment). Within each population, means accompanied by a different letter are significantly different (ANOVA and Newman-Keuls test on data transformed by arcsin ($\sqrt{y}$); $\alpha = 0.05$).

mone concentration. However, from 2 m to 1 m downwind of the lure, there was a significant reduction in net upwind flight speed of laboratory and lab–wild moths, but not wild moths, when Ald concentration was increased from 0.005% to 0.5% (Table 2). The net upwind flight speed of moths from 2 m to

TABLE 1. TEMPORAL RESPONSES OF WESTERN SPRUCE BUDWORM MALE MOTHS TO A RANGE OF CONCENTRATIONS OF 92 : 8 (*E/Z*)-11-TETRADECENAL (Ald), OR TWO VIRGIN FEMALE MOTHS, IN A FLIGHT TUNNEL. THE MOTHS WERE FROM THREE POPULATIONS: A LABORATORY COLONY (LAB), FIELD COLLECTIONS (WILD), AND LAB-WILD CROSSES. BECAUSE ANALYSIS OF VARIANCE SHOWED NO SIGNIFICANT DIFFERENCES AMONG POPULATIONS FOR THE VARIABLES BELOW, THE DATA WERE POOLED

	Time from release[a] to wing-fanning (sec)		Net upwind groundspeed of flight (cm/sec)[a]					
			1 m–0.5 m[b]		0.5 m–lure		UF[c]–lure	
Treatment	n	$\bar{x}$	n	$\bar{x}$	n	$\bar{x}$	n	$\bar{x}$
Female	57	8a	52	18a	50	11a	50	15a
0.5% Ald	80	7a	29	10b	25	7b	25	9b
0.05% Ald	59	9a	45	11b	40	7b	40	10b
0.005% Ald	36	27b	33	13ab	32	8b	32	11b
0.0005% Ald	13	35b	6	15	5	3	5	8
0.00005% Ald	5	35	8	13	8	7	8	11
Control	1	7	1	25	1	17	1	18

[a]Means followed by a different letter are significantly different (ANOVA and Newman-Keuls, $\alpha = 0.05$, data transformed by log $(y + 1)$). Data from concentrations $< 0.0005\%$ were not included in the analysis due to the low number of responders.
[b]Distance downwind from the lure.
[c]The onset of upwind flight.

TABLE 2. TEMPORAL RESPONSES OF WESTERN SPRUCE BUDWORM MALE MOTHS TO A RANGE OF CONCENTRATIONS OF SYNTHETIC 92 : 8 (E/Z)-11-TETRADECENAL (Ald), OR TWO VIRGIN FEMALE MOTHS, IN A WIND TUNNEL. THE MOTHS WERE FROM THREE STRAINS: A LABORATORY COLONY (LAB), FIELD COLLECTIONS (WILD), AND LAB-WILD CROSSES

	Time from release to taking-off (sec)[a]						Net groundspeed of flight from 2 m–1 m (cm/sec)[a,b]					
	Lab		Wild		Lab-Wild		Lab		Wild		Lab-wild	
Treatment	n	$\bar{x}$	n	$\bar{x}$	n	$\bar{x}$	n	$\bar{x}$	n	$\bar{x}$	n	$\bar{x}$
Female	33	28ab	30	13a	33	16a	19	18ab	18	22a	21	31a
0.5% Ald	30	24ab	29	31ab	29	23ab	9	7b	5	13a	15	21b
0.05% Ald	34	24a	31	19ab	33	21ab	18	12b	8	27a	24	21ab
0.005% Ald	29	38ab	28	32b	26	27ab	10	30a	8	18a	15	28a
0.0005% Ald	18	48ab	19	28ab	27	21ab	3	28			4	17
0.00005% Ald	19	52b	23	21ab	19	38b	3	33	1	20	3	12
Control	7	60ab	18	33b	10	40b			1	17		

[a] Differences among the three strains were significant (ANOVA, $\alpha = 0.05$) so the analysis was repeated separately for each strain. Means within columns followed by different letters are significantly different (ANOVA and Newman-Keuls, $\alpha = 0.05$; data transformed by log $(y + 1)$).
[b] Data from concentrations $\leq 0.0005\%$ were not included in the analysis due to the low number of responders.

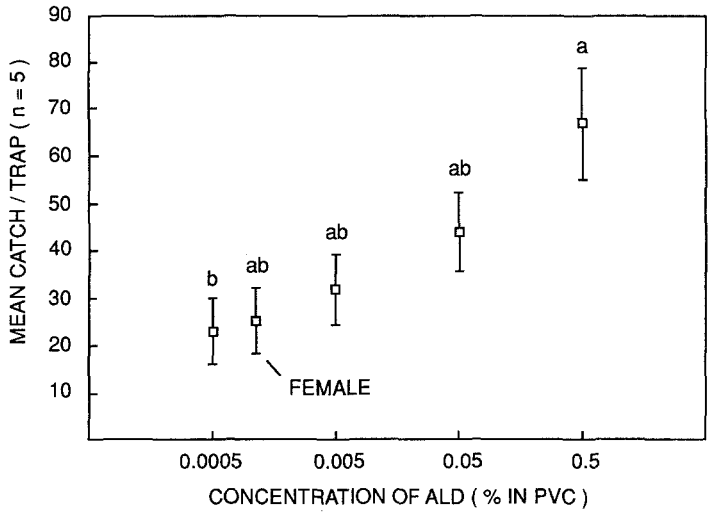

FIG. 5. Mean number of western spruce budworm moths caught in the field in sticky traps baited with a single virgin female moth or a range of concentrations of 92:8 (*E*/*Z*)-11-tetradecenal (Ald). Means accompanied by a different letter are significantly different (ANOVA and Newman-Keuls test on data transformed by log $(y + 1)$; $\alpha = 0.05$).

1 m downwind of the lure was significantly slower in the laboratory moths (16 cm/sec) than in the wild (21 cm/sec) or lab–wild moths (25 cm/sec) (ANOVA and Newman-Keuls, $\alpha = 0.05$; the 0.005%, 0.05% and 0.5% Ald treatments were pooled within strains).

The moths usually decreased their net upwind groundspeed as they approached within 30–40 cm of the lure. In all three populations, the net groundspeed from 0.5 m to the lure was significantly lower than that from 1 m to 0.5 m downwind, which in turn was significantly lower than that from 2 m to 1 m downwind from the lure (paired *t* tests, treatments within populations pooled, $P \leq 0.05$).

Field Bioassay. Trap catch increased with increasing Ald concentration, but the mean number of moths caught per trap was not significantly different between 0.005% and 0.5% concentrations (Figure 5). Traps baited with a virgin female caught a mean of only 25 moths, which was not significantly different from the number of moths caught in traps baited with any Ald concentration.

DISCUSSION

Percentage Response vs. Pheromone Concentration. The activation threshold for upwind anemotaxis of more than 10–15% of the test moths was between 0.0005% and 0.005% Ald (Figure 1). Also, although the difference

was not significant, fewer males completed upwind flight to the 0.5% lure than to the 0.05% lure, lending support to the hypothesis of an upper arrestment threshold (Roelofs, 1978). However, the number of moths caught in sticky traps placed in the field did not show a similar decrease when the Ald concentration was increased from 0.05% to 0.5% (Figure 5). We expected that traps baited with the highest pheromone concentration would have drawn moths from further downwind than traps baited with lower concentrations, but that a greater proportion of moths would have arrested flight prior to reaching the more concentrated lure, as Baker and Roelofs (1981) observed in the Oriental fruit moth. Baker et al. (1981) noted that the threshold concentration for arrestment of the Oriental fruit moth to pheromone-baited traps was about 10-fold higher in the field than what they had observed in a flight tunnel and suggested that the effective concentration of pheromone near a trap could have been reduced by greater turbulence in the pheromone plume.

No concentration of Ald elicited as much upwind flight and landing at the lure as did the virgin females. This supports other observations (Alford and Silk, 1984; Sweeney et al. 1990), which suggested that minor components in the female moth's effluvia, in addition to Ald, increased the percentages of upwind flight and lure contact. The catch in sticky traps baited with virgin females, relative to those baited with Ald, was lower than would be predicted from the flight-tunnel observations, but the traps were baited with only one female, whereas the flight-tunnel bioassays used two. We also have observed lower than expected response to virgin female moths in other sticky trap bioassays (Sweeney et al., 1990), and we suggest that high temperatures, contact with adhesive inside the trap, and the movement of trapped male moths may have possibly disrupted the calling behavior of the female moth.

Temporal Response vs. Pheromone Concentration. The onset of wing-fanning was significantly delayed in response to the lower pheromone concentrations. Cardé and Hagaman (1979) obtained similar results with the gypsy moth and suggested that the male moth begins wing-fanning when its central nervous system can distinguish a neuronal firing rate from background noise; this integration would require a longer period of stimulation at low than at high dosages. However, Linn and Gaston (1981), working with the cabbage looper, found no change in elapsed time from the exposure of a moth to pheromone and the onset of wing-fanning when they changed the pheromone concentration or blend.

All three strains of male moths reduced their net upwind groundspeed of flight as they approached the lure. This is consistent with results found in flight-tunnel studies of the spruce budworm (Sanders, 1985) and the Indianmeal moth, *Plodia interpunctella* Hübner (Marsh et al., 1978), and field studies of the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Murlis et al., 1982). Cardé and Hagaman (1979) suggested that the proximity of the calling female may be sensed by an increase in pheromone concentration detected by the male moth: as the male moth nears the lure, the plume narrows and mean concentra-

tion across the plume increases. The male responds by reducing his groundspeed and eventually arresting flight.

The Oriental fruit moth, *Grapholita molesta* (Busck), gauges its upwind progress by optomotor input and regulates its speed by maintaining a preferred image velocity (Kuenen and Baker, 1982a). As pheromone concentration increases, the optomotor response is modified, and the moth makes narrower and more frequent counterturns and eventually increases the angle of counterturns to 90° relative to a 0° wind axis, which arrests upwind progress (Kuenen and Baker, 1982b). Sanders (1986) suggested that male moths might regulate their net upwind groundspeed by selecting a preferred rate of flow of pheromone over their receptors instead of a preferred image velocity.

The hypothesis of an inverse relationship between net upwind groundspeed and pheromone concentration was supported only at distances of 2 m to 1 m downwind from the lure, and only in the laboratory and lab–wild males. The increased speed of net upwind flight in response to the virgin female moths, as compared with 0.05% Ald, suggests that the male's upwind progress was affected by minor components in the female's effluvia in addition to Ald. Sanders et al. (1981) found similar results with *C. fumiferana*.

Differences among Strains. The three strains of western spruce budworm moths showed significant differences in the percentages of wing-fanning and copulatory behavior, net upwind flight speed from 2 m to 1 m downwind, and in the antennal response to pheromone as measured on the EAG. These differences could be due to a low level of *Nosema* sp. infection in some of the laboratory moths. Heavy infection levels of *Nosema* sp. have been associated with reduced response of western spruce budworm moths to pheromone (Sweeney and McLean, 1987). Genetic drift may also have occurred in the laboratory population, since it has been bred in relative isolation for over 100 generations. Raina et al. (1989) attributed prolonged laboratory rearing as the cause of observed differences in the pheromone-mediated behavior of wild and lab-reared *Heliothis zea* (Boddie) male moths.

Our observation that the net upwind progress of laboratory moths was slower than that of wild moths may be related to the significantly slower mean recovery period of its antennae following pheromone stimulation (Figure 1B). It has been suggested that some moth species require an intermittent pheromone stimulus in order to maintain counterturning and upwind progress towards the pheromone source (Kennedy, 1983; Willis and Baker, 1984; Baker, 1986). Baker (1986) suggested that inflight arrestment may be due to saturation of the antennal receptors and the resulting fusion of neuronal fluctuations. The slow antennal recovery period of the laboratory moths may have resulted in earlier saturation of antennal receptors and slower upwind progress relative to the wild males.

The quantitative differences in behavior observed between the three populations indicates that caution must be used when applying results from studies

with laboratory insects to wild populations or from one wild population to another.

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EFFECT OF TRICHOME B EXUDATE OF *Solanum berthaultii* HAWKES ON CONSUMPTION BY THE COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* (SAY)

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Abstract—The leaf extract from *S. berthaultii* Hawkes (PI 473340) contains exudate from trichomes (type B). Consumption of *S. tuberosum* var. Norchip foliage by the Colorado potato beetle *Leptinotarsa decemlineata* (Say) was reduced when treated with the leaf extract. The leaf extract from a resistant *S. berthaultii* clone without type B trichome had no antifeedant activity. It suggests that more than one mechanism of resistance to the Colorado potato beetle exists in *S. berthaultii*.

Key Words—Colorado potato beetle, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, *S. berthaultii*, *S. tuberosum*, trichome, antifeedant, resistance.

INTRODUCTION

Solanum berthaultii Hawkes is resistant to several potato-infesting insects because glandular trichomes secrete an adhesive substance that entraps the insects (Gregory et al., 1986). On *S. berthaultii*, the development time, survival, and oviposition of the Colorado potato beetle were reduced (Casagrande, 1982; Groden and Casagrande, 1986; Wright et al., 1985), and entrapment of

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the small larval stages is not a factor for the lower biological performance of Colorado potato beetle (Dimock and Tingey, 1987). The level of resistance to Colorado potato beetle found in *S. berthaultii* was, however, correlated with the abundance of the trichome type B (Gregory et al., 1986; Casagrande, 1982; Tingey and Laubengayer, 1981), and resistance was attributed to the presence of toxic or antifeedant chemicals in the trichome type B exudate or in the leaf of *S. berthaultii*. The physical interference with movement by the trichome's pubescence and inhibition of feeding by high concentrations of glycoalkaloids in the foliage were not considered as important in conferring resistance (Groden and Casagrande, 1986).

Chemicals of different classes are produced by *S. berthaultii* trichomes (Ave and Tingey, 1986; Gregory et al., 1986; King et al., 1986, 1987a,b). Several of these have been shown to reduce or inhibit feeding in Colorado potato beetle. However, extrapolation on the antifeedant activity of other chemicals based on their chemical structure is not yet possible (Pelletier and King, 1987). The objective of this research was to study the effect of trichome type B exudate from *S. berthaultii* (PI 473340) on foliage consumption by adult Colorado potato beetles.

METHODS AND MATERIALS

Preparation of Leaf Extract and Trichrome B Exudate. Two *S. berthaultii* Hawkes (PI 473340) clones, grown from two seeds from the same plant and numbered 1726 and 1729 by Singh (1985) were used. The leaves of *S. berthaultii* 1726 bear trichomes of types A and B. *S. berthaultii* 1729 bears only type A trichomes. Plants were propagated from stem cuttings and grown in 20-cm pots in a greenhouse. They were fertilized weekly with Peters 20–20–20 formulation and used in the experiments when 4–8 weeks old.

To prepare leaf extract, leaves were dipped in methylene chloride for a few seconds, the solvent evaporated to dryness under vacuum at 35–40°C, and the waxes precipitated by the addition of acetone and removed from the solution by filtration (King et al., 1986). The acetone was evaporated to dryness, and the remaining material was then weighed and kept in absolute ethanol at −5°C. This procedure yielded 0.079 mg/cm^2 of leaf extract from *S. berthaultii* 1726 and 0.003 mg/cm^3 from *S. berthaultii* 1729.

The trichome B exudate was collected by gently pressing a clean glass microscope slide on both surfaces of a *S. berthaultii* 1726 leaf (Lapointe and Tingey, 1984). The exudate was then dissolved in absolute ethanol, filtered, and stored at −5°C in solution in absolute ethanol.

Insect Material. Adult Colorado potato beetles were collected during the 1986 and 1987 growing seasons from a one-acre field of *S. tuberosum* var.

Norchip located at The Pennsylvania State University Agricultural Research Center, Rock Springs, Pennsylvania. Dithane M-45 (2 lb/acre) was applied weekly to prevent blight infection. No insecticide was applied to the field during the two years.

The experiments with overwintered adult Colorado potato beetles were conducted between June 2 and June 10 in 1986 and May 21 and June 6 in 1987. Beetles were collected from the field and held in the lab for 24 hr on fresh *S. tuberosum* foliage prior to testing.

First-generation adult Colorado potato beetles were also tested. To obtain individuals of known age, fourth-instar larvae were collected and caged in the field until they pupated. Emerging adults were collected daily and transferred to another cage. Adult beetles were used two days after emergence and maintained during transportation to the lab and the preparation of the tests on fresh *S. tuberosum* foliage from the same field.

Bioassay. The no-choice bioassay consisted of placing one adult Colorado potato beetle in a 9-cm Petri dish for 2.5 hr at 29–31°C with one of the treatment leaflets. The five treatments were as follows: *S. tuberosum* (var. Norchip) leaflet treated with leaf extract from *S. berthaultii* clone 1726 or 1729; leaflet of *S. berthaultii* clone 1726 or 1729; and leaflet of *S. tuberosum* var. Norchip as control. The Petri dishes were lined with filter paper, and the side and top covered with fiberglass screening to prevent locomotion impairment of the males Colorado potato beetle (Pelletier and Smilowitz, 1987). Leaflets 8–12 cm^2 were collected from the top quarter of 4- to 5-week-old *S. tuberosum* plants grown outside the greenhouse in 20-cm pots in Metro Mix 200 soil mix and fertilized weekly with Peters 20–20–20 formulation. A Li-Cor leaf area meter (model LI-3000) equipped with a conveyer belt (model LI-3050A) was used to determine the *S. tuberosum* leaflet area. The leaf extract from *S. berthaultii* in absolute ethanol was evenly applied on the upper surface of the leaflet with a 0.25-cc tuberculin syringe (Yale model 2001) at 0.01 ml/cm^2. The control leaflets received absolute ethanol. After the ethanol evaporated, the leaflet was placed in a Petri dish with an adult Colorado potato beetle.

The dishes were placed on a lab bench during the experiment. Consumption was estimated by measuring the consumed part of the leaflet on graph paper. Frass on the leaflet prevented the use of the leaf area meter. Each replication consisted of eight controls and 16 treated leaflets; an equal number of male and female adult Colorado potato beetles were used. The trichome B exudate was tested using the same techniques as in the leaf extract but only with the first generation Colorado potato beetle in 1986.

The variation in feeding response to leaflets from *S. tuberosum* and *S. berthaultii* clones 1726 and 1729 was evaluated by repeating the test with the same individuals 24 hr after the first test. The insects were maintained in a Petri dish overnight and fed with fresh *S. tuberosum* foliage between tests.

The leaf thickness of the two plant species was estimated by comparing the fresh and dry weights of 10 leaf disks, 1.5 cm diam., from both plants species. Fresh *S. tuberosum* leaf disks were 8.8% heavier but 13.0% lighter when dry compared to *S. berthaultii* leaf disks. The data were not transformed.

Linear regression analyses were performed using the SAS REG procedure (SAS Institute, 1982). The overall average consumption for each treatment was used to calculate the dose–consumption curves for the leaf extract from *S. berthaultii* 1726. Means were compared using the t' test (Snedecor and Cochran, 1967).

RESULTS

The overwintered beetles consumed approximately half the amount of foliage of individuals of the first generation (Tables 1 and 2). Consumption of *S. tuberosum* leaflet treated with *S. berthaultii* 1726 leaf extract decreased linearly as the concentration of leaf extract increased. All the regressions were statistically significant (Table 3). The coefficients of determination (r^2) indicated that the concentration of leaf extract from *S. berthaultii* 1726 accounts for 72–88% of the observed variation in consumption. The regression slopes were lower for the males than for the females, indicating reduced effect of the leaf extract

TABLE 1. EFFECT OF LEAF EXTRACT FROM *S. berthaultii* CLONES ON CONSUMPTION OF *S. tuberosum* VAR. NORCHIP FOLIAGE BY OVERWINTERED ADULTS OF COLORADO POTATO BEETLE

Extract from	Conc. (mg/cm^2)	Consumption (mm^2)					
		Males			Females		
		Mean	SD	*N*	Mean	SD	*N*
Norchip		29.01	39.02	180	51.04	40.99	182
SB 1726	0.015	27.48	27.86	40	38.11	39.98	38
	0.03	19.92	29.24	48	54.83	38.30	48
	0.075	19.43	25.96	40	36.18	33.68	40
	0.115	13.10	20.21	52	36.75	37.73	52
	0.18	10.60	17.39	40	20.63	26.12	40
	Leaves	15.71	30.06	28	49.71	38.52	28
SB 1729	0.0075	21.09	28.40	32	31.75	28.08	32
	0.018	17.63	24.49	24	51.57	36.27	23
	Leaves	4.46	12.28	28	3.11	6.83	28

TABLE 2. EFFECT OF LEAF EXTRACT FROM *S. berthaultii* CLONES ON CONSUMPTION OF *S. tuberosum* VAR. NORCHIP FOLIAGE BY FIRST-GENERATION ADULTS OF COLORADO POTATO BEETLE

Extract from	Conc. (mg/cm^2)	Consumption (mm^2)					
		Males			Females		
		Mean	SD	*N*	Mean	SD	*N*
Norchip		80.02	47.12	165	87.34	50.52	173
SB 1726	0.015	61.03	37.50	40	83.45	40.22	40
	0.03	66.38	41.44	32	89.97	48.15	32
	0.075	62.78	33.26	32	64.88	44.18	32
	0.115	50.52	33.97	31	47.58	30.57	36
	0.18	38.65	27.60	34	52.28	34.28	36
	Leaves	42.47	54.33	70	49.58	59.21	79
Trichome B	Exudate	33.21	38.01	14	60.79	37.52	19
SB 1729	0.0075	63.00	44.52	16	94.13	44.11	16
	0.018	76.88	47.61	16	78.56	43.92	16
	Leaves	2.54	7.13	28	8.89	17.34	28

concentration on the males. The slopes of the dose–consumption curves were lower for both sexes for the overwintered generation than for the first generation of the beetle.

According to the dose–consumption curves, the consumption of *S. berthaultii* 1726 leaflet by males and females overwintered and first-generation beetles corresponded to the consumption of *S. tuberosum* leaflet treated with, respectively, 0.111 mg/cm^2, 0.002 mg/cm^2, 0.161 mg/cm^2, and 0.157 mg/cm^2 of *S. berthaultii* 1726 leaf extract. These concentrations are higher than the

TABLE 3. LINEAR REGRESSION ANALYSIS ON CONSUMPTION OF *S. tuberosum* LEAFLET BY ADULT COLORADO POTATO BEETLES TREATED WITH DIFFERENT CONCENTRATIONS OF *S. berthaultii* 1726 LEAF EXTRACT

Generation	Sex	Slope	Intercept	r^2	F^a
Overwintered	Male	−100.8	26.9	0.88	29.4**
	Female	−150.4	50.0	0.72	10.2*
First	Male	−189.1	73.0	0.85	22.6**
	Female	−242.1	87.7	0.81	16.8*

[a] Statistically significant at: $**P \leq 0.01$ and $*P \leq 0.05$.

amount of leaf extract per leaf area harvested from *S. berthaultii* 1726 (0.079 mg/cm^2) except for overwintered female adult Colorado potato beetles.

The consumption of *S. tuberosum* treated with leaf extract from *S. berthaultii* 1729 at concentrations of 0.0075 and 0.0180 mg/cm^2 was tested on adult beetles. These concentrations corresponded to 2.5 and six times the quantity normally extracted from a leaflet. The consumption of treated *S. tuberosum* foliage differed from the control for overwintered females at the 0.0075 mg/cm^2 concentration and for the males at the 0.0180 mg/cm^2 concentration. Except for these, no other differences were detected.

Bioassays of *S. tuberosum* leaflet treated with 0.075 mg/cm^2 of *S. berthaultii* 1726 trichome B exudate were performed on first-generation male and female Colorado potato beetle. The presence of trichome B exudate on *S. tuberosum* leaflet reduced the consumption of first-generation male beetles (Table 2) to a level comparable to the consumption obtained with concentrations of 0.115 and 0.180 mg/cm^2 of *S. berthaultii* 1726 leaf extract (t' test at $P \leq 0.05$). Similar results were obtained with the first-generation female adult beetles where the consumption on *S. tuberosum* leaflet treated with trichome B exudate was similar to the consumption of *S. tuberosum* leaflet treated with 0.075, 0.115, and 0.180 mg/cm^2 of *S. berthaultii* 1726 leaf extract (t' test at $P \leq 0.05$).

To determine the variability in the consumption by adult Colorado potato beetles, the bioassay was repeated with the same individuals after a 24-hr interval had elapsed. A linear relationship was found between consumption during the first and the second bioassay except for males on *S. berthaultii* 1729 and females on *S. berthaultii* 1726 (Table 4). When the correlation was significant, the relation between consumption during the two bioassays was relatively poor, with coefficients of determination (r^2) varying from 0.16 to 0.52.

TABLE 4. LINEAR REGRESSION ANALYSIS ON CONSUMPTION DATA OF ADULT COLORADO POTATO BEETLES DURING TWO BIOASSAYS WITH SAME INDIVIDUALS AT 24-hr INTERVAL

Sex	Plant	N	Slope	Intercept	r^2	F^a
Male	ST	31	0.47	52.59	0.30	12.62**
	SB 1726	50	0.74	10.24	0.52	52.01**
	SB 1729	27	0.16	6.38	0.01	0.13n.s.
Female	ST	34	0.41	78.56	0.16	6.21*
	SB 1726	60	0.19	37.38	0.05	3.04n.s.
	SB 1729	27	0.74	8.61	0.17	5.42*

[a] Statistically significant at: $**P \leq 0.01$, $*P \leq 0.05$; n.s. = statistically not significant.

DISCUSSION

Feeding in herbivorous insects is the consequence of several interdependent physiological, metabolic, and behavioral factors (Bernays, 1985; Hsiao, 1985). The internal conditions of the insect can influence several aspects of feeding behavior, such as locomotion, consumption rate, and sensitivity of chemoreceptors (Bernays, 1985; Hsiao, 1985).

The feeding tests indicate an antifeedant effect of the *S. berthaultii* 1726 on adult Colorado potato beetles (Tables 1 and 2). The slope of the leaf extract dose–consumption curve was inversely proportional to the consumption on *S. tuberosum* leaflet, indicating that the effectiveness of the leaf extract from *S. berthaultii* 1726 decreases as the consumption on the control decreases. Starvation can negatively affect the chemoreceptors and change the responsiveness to phagoactive chemicals (Bernays, 1985). Starvation is not a factor in this test since beetles were maintained on *S. tuberosum* foliage before the bioassays. On the other hand, Mitchell (1985) observed variation in sensitivity of some chemoreceptors in cohorts of adult Colorado potato beetles and speculated that environmental conditions during growth or other factors experienced by all members of the cohorts may have been responsible. The same conclusions can be used for the difference in the generations' reaction toward leaf extract from *S. berthaultii* 1726.

Variation in consumption on *S. tuberosum* and *S. berthaultii* by different individuals was also observed within the groups of Colorado potato beetle tested. Such a variation in consumption could result from differences in perception of the antifeedant or from variation of the internal conditions of the individuals. The consumption of several individuals varied between the two bioassays (Table 4). However, the average consumption by adults for each of the two bioassays was the same (t' test at $P \leq 0.05$), and results obtained with different plant species and leaf extract were reproducible at a population level.

Consumption of *S. berthaultii* 1726 was reduced compared to *S. tuberosum* leaflet treated with leaf extract equivalent to the quantity harvested from *S. berthaultii* 1726. Underestimation of the concentration of leaf extract on *S. berthaultii* is a possible reason for the difference. Furthermore, the exudate is more concentrated at the tip of the hair on *S. berthaultii* leaf, which could increase the effectiveness of the antifeedant. Contact of the concentrated droplet by the sensory organs influences the behavior of the Colorado potato beetle. The beetle requires only 1–2 sec to recognize and reject *S. berthaultii* 1726. Whereas when the extract is applied to *S. tuberosum*, it disperses over the leaflet surface and is not concentrated as it is on *S. berthaultii* (unpublished data). The droplets of exudate at the tip of a long hair constitute a barrier of concentrated feeding deterrent and may be more effective than the extract spread on the surface of the leaf.

Treatment of *S. tuberosum* leaflets with *S. berthaultii* 1726 trichome B exudate resulted in similar consumption as *S. tuberosum* leaflets treated with the same concentration of leaf extract from *S. berthaultii* 1726. This suggests that the trichome B exudate contains the active ingredient(s) found in the *S. berthaultii* 1726 leaf extract. It also suggests that other chemicals enhancing the antifeedant effect of the *S. berthaultii* 1726 trichome B exudate may also be present in *S. berthaultii* 1726 leaflet.

S. berthaultii 1729 does not bear trichome B and is more resistant to the Colorado potato beetle than *S. berthaultii* 1726. Leaf extract from *S. berthaultii* 1729 did not consistently reduce consumption compared to the control. Thin-layer chromatography reveals that only the band containing the trichome B exudate is missing from the *S. berthaultii* 1729 extract (Pelletier and Smilowitz, unpublished data; King et al., 1986). This supports the conclusion that the trichome B exudate is the active fraction of the *S. berthaultii* 1726 leaf extract and that the resistance of *S. berthaultii* 1729 is based on a different mechanism. Variation in the resistance level of *S. berthaultii* accessions to Colorado potato beetle had already been reported (Carter, 1987).

From the data gathered here, it is indicated that the genetic transfer of trichome B from *S. berthaultii* to cultivated potato varieties could reduce foliage consumption by the Colorado potato beetle. However, the protection provided by the *S. berthaultii* trichomes will vary with the beetle's generation, sex, and internal conditions. The growing conditions in the field may influence the quantity of trichome B exudate produced, and a qualitative variation of the trichome B exudate may exist between *S. berthaultii* accessions. More research is needed on the behavioral, genetic, and physiological aspect of *S. berthaultii* resistance toward Colorado potato beetle before the plant can be successfully used to incorporate long-lasting Colorado potato beetle resistance in potato.

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RELATION OF *Spodoptera eridania* CHOICE TO TANNINS AND PROTEIN OF *Lotus corniculatus*

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Abstract—Plant secondary compounds such as tannins may influence herbivore choice. To determine if herbivory was influenced by tannin concentration, *Spodoptera eridania* larvae were given a choice of *Lotus corniculatus* plants whose chemical profiles were altered by fertilization. Herbivores chose plants that had been grown with symbiotic nitrogen fixation as their only nitrogen source more often than fertilized plants. Choice was related to protein concentration, but not to tannin concentration.

Key Words—Condensed tannins, protein, *Spodoptera eridania*, Lepidoptera, Noctuidae, *Lotus corniculatus*, plant–insect interaction.

INTRODUCTION

Plant secondary chemicals may have a variety of effects on herbivores (Rosenthal and Janzen, 1979). For example, some herbivores may have increased growth when fed on tannins (Bernays et al., 1983), while others may be unaffected by tannins (Smiley and Wisdom, 1985; Fox and McCauley, 1977) or adversely affected by tannins (Rossiter et al., 1988; Feeny, 1970; Bryant et al., 1987). This spectrum of results appears to be related to how adapted on herbivore is to tannin (Berenbaum, 1983).

Manuwoto et al. (1985) determined that high-tannin diets fed to the generalist herbivore *Spodoptera eridania* Cramer (Noctuidae, southern armyworm) decreased larval feeding and consequently lowered larval growth. Scriber (1978) also noted that although cyanide in *Lotus corniculatus* L. (birdsfoot trefoil) did

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not affect choice of *S. eridania* larvae, larvae began to exert a choice late in the experiments, although the cause was not known. Since *L. corniculatus* produces condensed tannins (Jones et al., 1973) in addition to cyanogenic glycosides, and tannin concentration increases as plants grow older and larger (Briggs, 1990), it seems possible that tannin concentration of *L. corniculatus* may have affected choice of *S. eridania*.

I report here the results of experiments that relate *S. eridania* choice among *L. corniculatus* plants whose chemical concentrations of condensed tannins and protein were altered by the addition of nitrogen fertilizer.

METHODS AND MATERIALS

Plants. Experimental plants were obtained from a stock population of *L. corniculatus* started from seed in 1985 and maintained until 1987. In early May 1987, nine 13- to 15-cm shoot cuttings (replicates) were made from each of 14 randomly selected genetic individuals (genotypes). Cuttings were labeled, treated with rooting hormone, and placed in sand under mist. After roots formed, plants were inoculated with *Rhizobium* and transplanted to 4.4-liter pots containing soilless mixture lacking nitrogen (peat moss, perlite, vermiculite, triple phosphate, dolomite limestone, and micronutrients) in early June. Pots were placed on plastic sheets within a fenced (chicken wire) enclosure in an open field located in University Park, Pennsylvania. Plants were watered daily throughout the experiment.

Three replicates per genotype were distributed among three fertilization treatments (126 plants). Fertilization began on June 23, three weeks after transplanting, and continued until plants were harvested for choice tests; July 26 was the last harvest. Plants in one treatment (Zero N) obtained nitrogen solely from symbiotic nitrogen fixation. Plants in the other two treatments were fertilized five days a week throughout the experiment. Each plant in the low N treatment received approximately 0.03 g of ammonium nitrate (in solution) five days a week, while high N plants received approximately 0.13 g. Excess solution commonly flowed from the pots.

Insects and Choice Tests. S. eridania eggs were generously donated by the FMC Corporation of Princeton, New Jersey. Eggs began hatching on July 13. Hatchlings not used immediately for choice tests were placed on a wheat-germ diet (120 g wheat germ, 33 g sucrose, 25 g casein, 8 g salt, 2 g sorbic acid, 1 g methyl paraben, 10 g vitamin premix, 15 g agar, and 800 ml water).

To determine if any chemical measures affected insect choice, all nine replicates of an *L. corniculatus* genotype (three replicates × three N treatments) were brought into the lab, and any flowers and fruits were removed. From each fertilization treatment, all three replicates were clipped at the root–shoot junc-

tion and immersed in water; 10 stems were recut underwater to approximately 15 cm in length and inserted into Aqua-pics. Excess shoots (leaves and stems) were flash-frozen in liquid nitrogen, freeze-dried, and stored at −30°C until analyzed. Three cuttings, one from each fertilization treatment, were taped in alternating patterns to the same wall in each of 10 transparent plastic chambers (12 × 17 × 31 cm), and disinfected with 10% Clorox before use. Ten larvae were removed from artificial diet, and one larva was added to each chamber. Larvae were only tested once. Chambers were kept in a 23°C, 16 hr day–8 hr night growth chamber.

Two plant genotypes were tested simultaneously over a 45-hr period in each of seven trials between July 14 and 26, utilizing first to fourth instar larvae. The cutting (zero N, low N, or high N) that an herbivore was eating was noted three times over the 45-hr period. If a larva fed on more than one cutting (a common occurrence), larval choice was visually scored at test termination as the cutting that sustained the most damage. Cuttings were never completely defoliated. For every plant genotype–fertilization treatment combination, choice was designated as the number of larvae, out of 10 replicates, which mostly ate cuttings of that treatment.

Chemical Analyses. Because choice tests were set up to determine within-genotype choice, cyanide concentration was not determined. The cyanide concentration in *L. corniculatus* rarely varies between replicates of a genotype, regardless of fertilization treatment (Briggs, 1990). Scriber (1978) also found that *L. corniculatus's* cyanide did not influence *S. eridania* choice.

Freeze-dried shoot material was ground and a subsample was removed and extracted in 70% acetone for tannin quantification [vanillin or leucoanthocyanidin (LA); Folin-Denis or total phenolics; and hemoglobin binding or astringency assays; Schultz and Baldwin, 1982; Schultz et al., 1981]. A subsample of shoot material also was analyzed for protein content using a microprotein assay (Bradford, 1976; Compton and Jones, 1985)

Statistical Analyses. General Linear Models (GLM) (SAS, 1982) were used to determine if insect choice was influenced by fertilization treatment, time of test, or any chemical measures. GLM was also used to determine if fertilization treatment was related to tannin or protein concentrations. When fertilization was significant main effect, Tukey's HSD (SAS, 1982) was used to determine how treatments differed.

RESULTS

Plant Chemistry. Plants grown under the different fertilization treatments differed in LA ($P = 0.04$) and total phenolics ($P = 0.001$, Figure 1) but did not differ in astringency ($P = 0.19$). Low N plants had higher LA concentra-

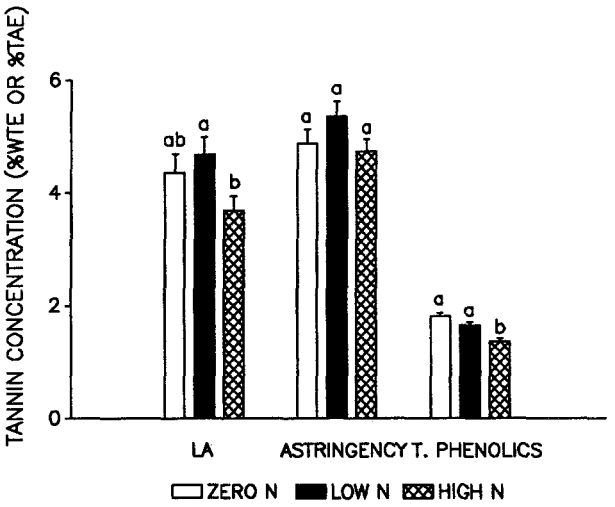

FIG. 1. The influence of nitrogen source on leucoanthocyanidins (LA), measured as percent wattle tannin equivalents (% WTE); astringency, measured as percent tannic acid equivalents (% TAE); and total phenolics (T. phenolics), measured as % TAE, of *Lotus corniculatus*. Mean tannin concentration plus standard error. Different letters within an assay indicate significant differences between treatments ($P < 0.05$).

tions than high N plants, while LA concentration in zero N plants was not different from either of the fertilized treatments. Total phenolics followed the same basic trend, except that both low N and zero N plants had higher total phenolics than high N plants.

Plants grown under different fertilization treatments also differed in protein concentration ($P < 0.01$, Figure 2). Zero N plants had higher protein concentration than low N plants. Protein concentration of high N plants was not different from either the zero N or low N plants. Differences in protein and tannin concentrations may be related, in part, to altered allocation to growth, reproduction, and nitrogen fixation in the different fertilization treatments (Briggs, 1990).

Choice. Choice of *S. eridania* was not influenced by time ($P = 1.00$), but larval choice was different among fertilization treatments ($P = 0.01$, Figure 3). Larvae chose to eat zero N plants more often than low N plants ($P < 0.05$). Larval choice of high N plants was not different from either of the other treatments ($P > 0.05$). When larval choice was examined in relation to plant chemistry, *S. eridania* choice was associated with protein concentration ($P = 0.01$), but not with astringency, LA, or total phenolics ($P > 0.80$).

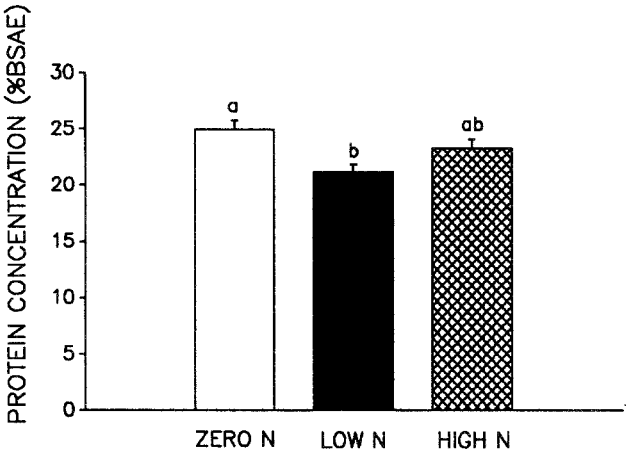

FIG. 2. The influence of plant nitrogen source on protein concentration, measured as percent bovine serum albumin equivalents, of *Lotus corniculatus*. Mean plus standard error. Different letters indicate significant differences between treatments ($P < 0.05$).

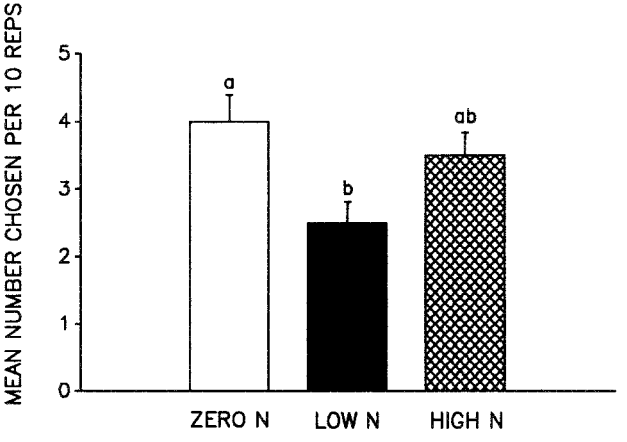

FIG. 3. *Spodoptera eridania* choice of stem cuttings from *Lotus corniculatus* plants of a single genotype whose propagules were grown under three nitrogen treatments. Mean choice (plus standard error) of 10 choice test replicates (reps) for each plant genotype; $N = 14$ genotypes. Different letters indicate significant differences between treatments ($P < 0.05$).

DISCUSSION

Time did not influence the outcome of choice tests. This suggests that choice was not influenced by either any seasonal fluctuations in plant chemistry or larval age.

Because Manuwoto et al. (1985) saw a decrease in food consumption and/or growth rates of *S. eridania* fed on high-tannin diets, I expected *S. eridania* larvae would choose low-tannin plants more often. Contrary to my hypothesis, tannin concentrations were not associated with herbivore choice in this experiment. The nondeterrence of tannins may be related to several factors. First, although LA and total phenolics varied among treatments, plants in the different fertilization treatments did not differ in astringency (Figure 2). While the LA and total phenolic assays measure structural aspects of tannins, the astringency assay is a measure of the protein-binding ability of tannins that may render plant tissue unpalatable to herbivores (Bate-Smith, 1973). Astringency is known to deter vertebrate herbivores (Oates et al., 1977; Woodruff et al., 1982). Because there was no difference in astringency, larvae may not have detected any tannin-based differences in the offered choices. Therefore, there may have been no basis for food choice.

Second, tannin levels may have been so low in experimental plants that larval choice was not affected. Although the experiments by Manuwoto et al. (1985) did not measure astringency, they included approximately 7% condensed tannins in *S. eridania* diets. *L. corniculatus* plants used in this experiment contained approximately 4.5% condensed tannins. Without measures of astringency, it is dangerous to compare tannins from different sources, but it is possible that the larvae in the Manuwoto et al. (1985) experiments experienced relatively higher tannin levels.

Larval choice was related to protein concentration. Larvae chose to eat zero N plants, which typically contained higher protein concentrations, more often than they chose plants in either the low N or high N treatments. While it is possible that other, unquantified, compounds may have influenced herbivore choice, protein concentration appears to have a major influence on *S. eridania* choice at low-tannin concentrations. Nitrogen content of food has been shown to have a greater influence on herbivores than tannins in several other systems (e.g., Fox and Macauley, 1977; Smiley and Wisdom, 1985; Yokoyama and Mackey, 1987).

These results suggest that although tannins are known to depress *S. eridania* food consumption (Manuwoto et al., 1985), the low levels of *L. corniculatus* tannins examined in this experiment did not protect plants from this herbivore, and susceptibility to herbivores appears to increase as protein concentrations increase. It is likely that plants with relatively higher protein levels

may suffer more damage as long as tannin concentrations are too low to influence herbivore choice.

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INFLUENCE OF TOBACCO LEAF SURFACE CHEMICALS ON GERMINATION OF *Peronospora tabacina* ADAM SPORANGIA

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Abstract—Chromatographic procedures were utilized to isolate and purify components of tobacco cuticular extracts and leaf surface chemicals. *In vitro* microbial bioassays determined the influence of these leaf surface compounds on germination and germ tube morphology of *P. tabacina* sporangia, the tobacco blue mold pathogen, and to a lesser extent *Alternaria alternata*, the tobacco brown spot pathogen. Exposure to 10 $\mu g/cm^2$ of α- and β-duvatrienemonols, sucrose esters, or hydrocarbons did not inhibit germination, whereas germination was significantly decreased by *cis*-abienol. *cis*-Abienol did not inhibit sporangial germination when combined with sucrose esters or hydrocarbons at a combined 10 $\mu g/cm^2$. Germination of sporangia was completely inhibited by α- and β-duvatrienediols. In contrast to a previous report, α-DVT-diol was more inhibitory than the β isomer. Toxic effects of the DVT-diols were not altered by pH. Diluting the DVT-diols to less than 0.1 $\mu g/cm^2$ resulted in a small but significant stimulation of germination. Previously, the DVT-diols had been identified only as inhibitory to *P. tabacina*. None of the leaf surface chemicals affected germination of *A. alternata* conidia.

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Key Words—*Nicotiana,* Solanaceae, *Peronospora tabacina,* blue mold, *Alternaria alternata,* leaf surface chemistry, diterpenes, sucrose esters, hydrocarbons, spore germination.

INTRODUCTION

Trichome exudates of *Nicotiana tabacum* L. may contain sucrose esters [(6-*O*-acetyl-2,3,4-tri-*O*-acyl)-α-D-glucopyranosyl-α-D-fructofuranosides], wax esters, fatty alcohols, α- and β-4,8,13-duvatriene-1-ols (DVT-monols), 12, *Z*-labda-12,14-diene-8α-ol (*cis*-abienol), and α- and β-4,8,13-duvatriene-1,3-diols (DVT-diols) (Roberts and Rowland, 1962; Chang and Grunwald, 1976; Severson et al., 1984). Hydrocarbons, which are not produced by trichomes, are also major components of tobacco cuticular extracts (Severson et al., 1984). Not all leaf surface constituents are found in every tobacco variety. Typical levels of leaf surface components after analysis of more than 30 tobacco varieties were in the range of <1–4 $\mu g/cm^2$ of DVT-monols, 5–18 $\mu g/cm^2$ of sucrose esters, 7–12 $\mu g/cm^2$ of *cis*-abienol, 11–16 $\mu g/cm^2$ of total hydrocarbons, and 2–60 $\mu g/cm^2$ of DVT-diols (Severson et al., 1985b). The DVT-diols, which are unique to *Nicotiana,* are the major diterpene components of leaf wax in many tobacco varieties (Gamou and Kawashimi, 1979). Leaf cuticular components, especially the DVT-diols, were implicated in resistance of plants to pests (Johnson and Severson, 1982; 1984; Cohen et al., 1983). Resistance by tobacco to insects such as tobacco budworm (*Heliothis virescens* F.), and green peach aphid (*Myzus persicae* Sulzer) was associated with levels of DVT-diols and DVT-monols on the leaf surface (Johnson and Severson, 1982; 1984). Several diterpenes, including the DVT-diols also have fungicidal properties (Bailey et al., 1974; Cruickshank et al., 1977; Cohen et al., 1983). Sclareol and 13-episclareol, two labdane diterpenes isolated from *N. glutinosa,* inhibit mycelial development of several fungi (Bailey et al., 1974). Another labdane diterpene, 2-ketoepimanool isolated from *N. glutinosa*, inhibits germination of *Erysiphe cichoracearum,* the fungal pathogen that causes powdery mildew of tobacco (Cohen et al., 1983). The DVT-diols inhibit germination of *P. tabacina,* the obligate fungal pathogen responsible for tobacco blue mold disease (Sheperd and Mandryk, 1963; Cruickshank et al., 1977; Menetrez et al., 1987). Tobacco plants are most susceptible to *P. tabacina* in the plant bed and during early plant growth when the concentrations of DVT-diols on the leaves are low (Severson et al., 1985b). Removal of DVT-diols from tobacco leaf surfaces can increase the plant's susceptibility to blue mold disease (Reuveni et al., 1987). The effect of other leaf surface components on *P. tabacina* has not been previously examined. Prior to this study, chemicals have been examined individually without taking into account the presence of other chemicals and their

potential to interact through various mechanisms with pathogens on the leaf surface. Our objective was to determine the influence of all major tobacco leaf surface chemicals on germination and germ tube morphology of *P. tabacina* sporangia *in vitro*. We examined some potential interactions of leaf surface chemicals as a first step in approaching the complexities that exist on leaf surfaces and that have been ignored by all previous studies. For comparative purposes, conidia of *A. alternata* (Fries) Keissler, the fungal pathogen causing tobacco brown spot, were similarly treated to determine the general nature of the effects of the leaf surface chemicals on a different fungal leaf pathogen.

METHODS AND MATERIALS

Inoculum Production. Tobacco (*Nicotiana tabacum* L. cv. Speight G-28) plants were grown under greenhouse conditions in Hyponex All Purpose Potting Soil (Hyponex Corporation) in 10-cm-diam. clay pots. Plants in the five- to six-leaf stage were sprayed to run-off with an aqueous suspension of freshly harvested sporangia (10^4/ml) of *P. tabacina* isolate OPT-1 (Spurr and Todd, 1982). Inoculated plants were kept at 18°C, in darkness and 100% relative humidity (RH) for 12 hr and then transferred to 20°C and 80% RH with a 12-hr photoperiod. Chlorotic lesions developed after seven to eight days, at which time plants were placed overnight in darkness at 18°C and 100% RH to induce fungal sporulation. Sporangia were removed from the leaves with a minimum amount of deionized water, filtered through six layers of cheesecloth, and counted in a hemacytometer cell. *A. alternata* isolate A5 was grown on Difco potato dextrose agar (PDA) for 14 days at 25°C with an 8-hr photoperiod. Conidial suspensions were obtained by flooding the Petri dish surface with deionized water and gently releasing the conidia with a spatula. The suspensions were filtered through six layers of cheesecloth and used immediately after adjusting the concentration.

Isolation of Crude Leaf Surface Mixture. Whole, young, green leaves of *N. tabacum* L., Brazilian domestic variety, Galpao, were extracted by immersion for 1-min in methylene chloride (CH_2Cl_2). Galpao was chosen because it contains high quantities of all of the major tobacco leaf surface constituents (Figure 1). The crude extract was filtered through Whatman No. 1 filter paper under aspirator vacuum and taken to near dryness at 35°C in a rotary evaporator. The final concentrated extract was dried over sodium sulfate and stored in an amber bottle under nitrogen atmosphere at −80°C until needed.

Preparative Chromatography. An aliquot of crude extract (ca. 300 mg/ml) was filtered through a 0.45-μm, 25-mm nylon 66-disk filter, and then injected onto a 2.5 × 68.5-cm bed of Sephadex LH-20. Chromatography was effected isocratically under lobar conditions at 5 ml/min using a 99:1 CH_2Cl_2–MeOH

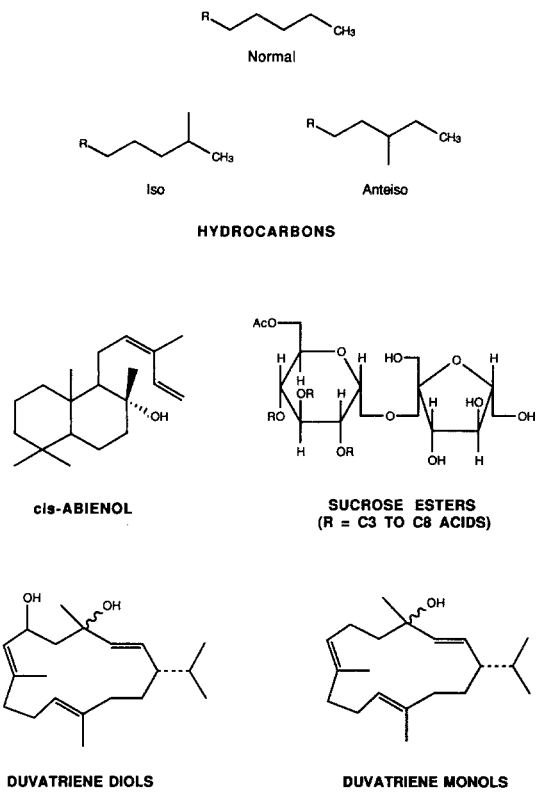

FIG. 1. Chemical structures of leaf surface chemicals assayed for toxicity to germination of *P. tabacina* sporangia.

mobile phase. Fractions (5 ml) were collected and subjected to thin-layer chromatography (TLC) on precoated silica gel plates that had been predeveloped twice in 50:50 CH_2Cl_2–MeOH and then activated. For visualization, dried plates were evenly sprayed with 10% ethanolic phosphomolybdate and then heated in an oven at 100°C for 1–3 min or until visualization was optimized. Fractions having like composition according to TLC results were combined, evaporated to dryness at 30°C under aspirator vacuum, and transferred to amber vials. Samples were stored under nitrogen atmosphere at −80°C until needed. More definitive identification of combined fractions was obtained using gas chromatography (GC). Combined fractions (1 mg) were blown to dryness under a stream of nitrogen at 40°C. Dried samples were then derivatized with 50 μl of 1:1 BSTFA–DMF (*N,O*-bis(trimethylsilyl)trifluoroacetamide–dimethylformamide) for 35 min at 75°C under an atmosphere of nitrogen. After the samples

had cooled, 50 μl of 1 : 1 BSA–pyridine (*N,O*-bis(trimethylsilyl)acetamide–pyridine) was added. Derivatized samples were subjected to GC analysis using a Varian 3700 GC equipped with a 20-m × 0.53-mm-ID DB-5 fused-silica column. The injector and flame ionization detector temperatures were 180 and 300°C, respectively. The column temperature was programmed from 160 to 300°C at 4°C/min. Helium was used as the carrier gas at a linear gas velocity of 28 cm/sec. Peak identification was based on the retention times of authentic samples and GC-MS analysis.

Separation of α- and β-DVT-diols. A sufficient aliquot of the crude, isomeric mixture (ca. 200 mg/ml) of α- and β-DVT-diol was filtered through a 0.45-μm, 25-mm nylon 66-disk filter, and injected onto a 2.5 × 21.5 cm column bed of silica gel (230–400 mesh). Chromatography was effected isocratically at 5 ml/min using a 1 : 1 : 8 isopropanol–chloroform–hexane (IPA–$CHCl_3$–HEX) mobile phase. Eighty fractions (5 ml) were collected in 1.5 × 15-cm test tubes.

TLC and GC analyses were carried out in the same manner as described above. TLC plates were developed in 1 : 1 : 8 IPA–$CHCl_3$–HEX. Separation of the DVT-diol mixture afforded both isomers in a purity >98% by GC analysis.

Separation of cis-Abienol–DVT-monols. Chromatographic procedures were the same as those described above for the DVT-diols with the following exceptions. One milliliter of an approximately 200 mg/ml solution of *cis*-abienol–DVT-monols mixture was injected and isocratically eluted with 7 : 3 CH_2Cl_2–HEX at 4 ml/min. TLC plates were developed in 7 : 3 CH_2Cl_2–HEX. Purity of the *cis*-abienol fraction was 95% by GC. The DVT-monol mixture was 98% pure and consisted of a mixture of the α and β isomers in roughly a 1 : 2 ratio.

Sucrose Ester Isolation. Fractions obtained from the Sephadex LH-20 wash with 97 : 3 CH_2Cl_2–CH_3OH that contained a mixture of sucrose ester isomers were pooled (Severson et al., 1985a). Purity was >95% by gas chromatographic analysis.

Stock solutions (2 mg/ml) of each of the leaf surface components and all dilutions were made with CH_2Cl_2.

Microbial Bioassay. Chemical solutions (50 μl) were evenly applied on the surface of 2 ml of solid 1.5% water agar (WA), pH 6.5, in 35 × 10-mm Petri dishes. Chemicals were delivered to the center of the agar plate and uniformly distributed over the surface of WA with the smooth surface of a round, Teflon-coated stir bar. Solvent was evaporated, and an aqueous suspension (300 μl) of freshly harvested *P. tabacina* sporangia or *A. alternata* conidia (1×10^5/ml) was added. When chemicals were combined for an assay, a 1 : 1 mixture of each component was added to the WA to make a combined concentration of 10 $\mu g/cm^2$. Biological activity was assessed by determining germination on duplicate samples after incubating 4 hr at 18°C and comparing to both CH_2Cl_2 and water controls. Methylene chloride controls consisted of agar plates in which

50 μl of CH_2Cl_2 was added and then allowed to evaporate. Germination of sporangia on these plates was calculated to determine effect of any CH_2Cl_2 residue on germination. If the solvent was not allowed to evaporate prior to addition of spore suspension, zero germination occurred. Data were reported as the mean of three or more replications.

Responses to pH. Various buffers including sodium acetate, sodium phosphate, glycylglycine, MES [2-(*N*-morpholino)ethanesulfonic acid] and HEPES (*N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) over the pH range of 3.5–8.5 were tested for their effects on germination of sporangia on WA. Since these buffers inhibited germination, bioassays to test the effect of pH on chemical activity were performed on PDA. PDA pH was adjusted with HCl or NaOH and maintained the desired pH without additional buffers. The nutrients in PDA did not influence germination. Agar pH was adjusted at 50°C and verified at room temperature (25°C) with a combination flat surface electrode (Fisher Scientific). Chemicals (10 $\mu g/cm^2$) were applied and tested for activity as described above.

RESULTS

Germination Responses to Individual Compounds. Germination was initially determined after exposure of *P. tabacina* sporangia to DVT-diols, DVT-monols, *cis*-abienol, sucrose esters, and hydrocarbons (Figure 1) at a concentration of 10 $\mu g/cm^2$ (Table 1). Germination was 80% in both water and CH_2Cl_2 controls. Exposure of sporangia to α- and β-DVT-diols totally inhibited germination. At this initial concentration, no difference in activity was found between the two DVT-diol isomers. DVT-monols, sucrose esters, and the hydrocarbon fractions influenced germination minimally. Germ tube lengths and sporeling physical appearance were similar to the controls. Germination of *P. tabacina* sporangia exposed to 10 $\mu g/cm^2$ *cis*-abienol was significantly inhibited. Germ tubes were normal in appearance but were one third the size of controls.

Germination Responses to Combined Compounds. P. tabacina sporangia were exposed to a mixture (10 $\mu g/cm^2$) of hydrocarbons and sucrose esters (HC + SE) and observed for synergism in stimulation of germination (Table 1) (see Methods and Materials). Synergism was not observed. Percent of germination (72%) after exposure to the combined fractions was similar to germination levels of sporangia exposed to the individual chemicals (73% and 71%, respectively). Combining *cis*-abienol with either the sucrose esters (CAB + SE) or the hydrocarbon fraction (CAB + HC) eliminated the inhibitory activity that *cis*-abienol alone had on *P. tabacina* germination. The limited amount of DVT-monols isolated prevented us from testing it in combination with other leaf

TABLE 1. GERMINATION OF *Peronospora tabacina* SPORANGIA AFTER EXPOSURE TO LEAF SURFACE CHEMICALS AT 10 $\mu g/cm^2$[a]

Chemicals	Germination (%)
α-DVT-diol	0.1[b]
β-DVT-diol	0.0[b]
α- and β-DVT-diol	0.0[b]
Monols	68 ± 5
Sucrose esters (SE)	71 ± 1
Hydrocarbon (HC)	73 ± 2
cis-Abienol (CAB)	43 ± 7
HC + SE[c]	72 ± 4
CAB + SE[c]	73 ± 5
CAB + HC[c]	72 ± 4
Methylene chloride	80 ± 4
Water	80 ± 3

[a] Each data point represents the mean (±SE) of three replicates.
[b] Combinations of leaf surface components with the DVT-diols resulted in zero germination.
[c] 5 $\mu g/cm^2$ of each component.

surface components. The ability of DVT-diols to completely inhibit germination was not eliminated when the DVT-diols were combined with any of the other leaf surface components (data not shown).

Conidia of *A. alternata* were also exposed to extracts (10 $\mu g/cm^2$) of leaf surface chemicals. *A. alternata* germination rates were greater than 90% after exposure to the leaf surface chemicals, including the DVT-diols. Germ tube morphology was not altered. Further growth and development of *A. alternata* in the presence of the chemicals was not evaluated.

Responses to Changes in pH. Leaf surfaces of tobacco grown in the greenhouse has a pH range of 5–8.5, whereas in the field it is 6–8 (Menetrez and Spurr, unpublished data). The effects of changes in pH on *P. tabacina* germination and toxicity of the diterpenes was assayed. *P. tabacina* sporangia germinated over a broad pH range (Figure 2). Germination did not occur at pH 3.5 through pH 4.5 but began at pH 5. Germination increased considerably at pH 5.5, reaching a plateau that continued to pH 8.5, These data agree with the optimum pH range obtained by Shepherd (1962) when he suspended *P. tabacina* sporangia in 0.01 M phosphate-citrate buffer and germinated them on Difco Bacto agar. Toxic effects of the DVT-diols on germination were not influenced by changes in pH (Figure 2). Germination levels were greater for sporangia exposed to *cis*-abienol on PDA plates pH 6–8 (Figure 2) than sporangia exposed to *cis*-abienol on WA plates (pH 6.5) (Table 1). The percent inhibition relative

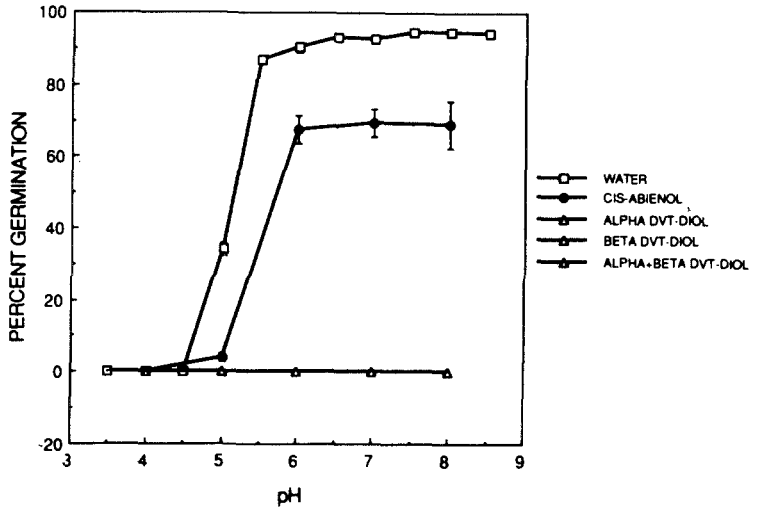

FIG. 2. Effects of pH (4–8) and leaf surface chemicals (10 $\mu g/cm^2$) on germination of *Peronospora tabacina* sporangia. Each data point represents the mean of three replicates. Bars indicate the standard error of the mean. Bars not shown where height of icon exceeds length of bar.

to the controls varied by 9% with 37% inhibition on PDA and 46% inhibition on WA. The data obtained from the PDA plates was also more variable at these pH ranges.

Dilution Effects on Activity of Chemicals. The effect of leaf surface chemical concentration on inhibition of germination of *P. tabacina* sporangia is shown in Figures 3A–C. The two controls, water and CH_2Cl_2, are represented by the zero baseline. Areas above the baseline represent inhibition of germination and areas below the baseline represent stimulation. As reported earlier, *cis*-abienol at 10 $\mu g/cm^2$ significantly inhibited germination, while the hydrocarbons and sucrose esters showed only slight inhibitory activity (Table 1, Figure 3A). Germination percentage, however, was enhanced 10% above the controls as the chemicals were diluted. Stimulatory activity of the three surface components began to plateau at 0.01 $\mu g/cm^2$. Inhibition of germination by α-DVT-diol and the two DVT-diol isomers in combination decreased rapidly from 100% inhibition at the initial concentration of 10 $\mu g/cm^2$ to only 10% inhibition at 1 $\mu g/cm^2$ (Figure 3B). Toxic effects of β-DVT-diol on germination decreased from 100% inhibition at 10 $\mu g/cm^2$ to zero inhibition at 1 $\mu g/cm^2$. As the concentration of all three chemicals decreased below 0.1 $\mu g/cm^2$, germination was stimulated.

Twofold dilutions of the DVT-diols (Figure 3C) were done to verify the precipitous change in chemical activity observed in Figure 3B. The inhibitory activity of α-DVT-diol remained high through 5 $\mu g/cm^2$ but decreased rapidly

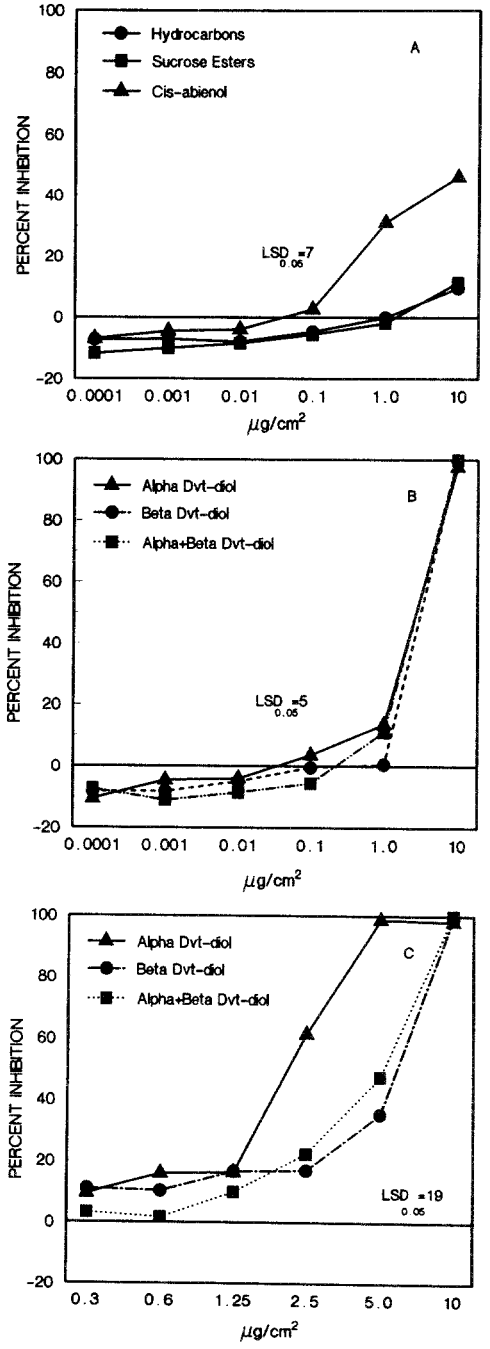

FIG. 3. Percent inhibition of germination of *Peronospora tabacina* sporangia relative to controls after exposure of sporangia to various concentrations of tobacco leaf surface chemicals. (A–B) 0.0001–10 $\mu g/cm^2$, (C) 0.3–10 $\mu g/cm^2$. Each data point represents the mean of three replicates. Analysis of variance was used to calculate least significant difference.

as it was diluted further. β-DVT-diol and the DVT-diols in combination began to lose inhibitory activity at the initial twofold dilution.

DISCUSSION

Tobacco leaf surface constituents probably influence the resistance of the plant to blue mold disease. Shepherd and Mandryk (1963) found that leaf surface concentrations of the two DVT-diol diterpenes were adequate to protect tobacco plants from fungal attack. They concluded that significant protection against blue mold disease did not occur because of uneven distribution and/or leaching of the compounds. Results of our microbial bioassays agree in part with their results (Shepherd and Mandryk, 1963; Cruickshank et al., 1977) and demonstrate additional activity not reported to date. Inhibition of germination of *P. tabacina* sporangia by the DVT-diols was confirmed. However, we found that as the DVT-diols were diluted, germination was stimulated. Prior to this study, the DVT-diols had been identified only as inhibitory to *P. tabacina* (Shepherd and Mandryk, 1963; Cruickshank et al., 1977). Cruickshank et al. (1977) reported the ED_{50} (μg/ml) of α- and β-DVT-diol as 24 and 17, respectively. Contrary to their report, our data (Figure 3B) suggested that the α isomer is more toxic. *cis*-Abienol, a diterpene like the DVT-diols, also had significant biological activity. On WA at 10 $\mu g/cm^2$, *cis*-abienol inhibited germination by 46%. Interpolation of germination percentages between 1 and 10 $\mu g/cm^2$ (Figure 3A) estimate that exposure of sporangia to *cis*-abienol at 5 $\mu g/cm^2$ would result in 40% inhibition of germination. Sporangia exposed to a 1:1 mixture (10 $\mu g/cm^2$ total) of *cis*-abienol and hydrocarbons (CAB + HC) or sucrose esters (CAB + SE) germinated at 72 and 73%, respectively (Table 1). These results indicate that *in vitro* interactions between *cis*-abienol and other leaf surface components decrease the inhibitory activity of *cis*-abienol and that the effects are not solely due to dilution.

Sucrose esters have shown antimicrobial activity against gram-positive bacteria such as *Bacillus subtilis* but are inactive against gram-negative bacteria and the fungi *Curvularia lunata* and *Asperigllus flavus* (Cutler et al., 1986). Sucrose esters were also ineffective in our studies. Prior to this study, the hydrocarbon fraction of leaf surface constituents had not been assayed for biological activity. Contact of *P. tabacina* sporangia with hydrocarbons did not influence germination. Possible interactions of hydrocarbons with leaf surface components, however, should continue to be investigated.

Genetic factors, physiological status, cultural practices, and environment play key roles in determining leaf surface chemistry (Severson et al., 1985b). The amount of DVT-diols, monols, sucrose esters, and *cis*-abienol in six different tobacco cultivars was significantly reduced after a 3.3-cm rainfall (Severson et al., 1985b). Rate of removal was not dependent on polarity. The

monols, the least polar, were removed at a similar rate to that of the sucrose esters, the most polar component.

Wind-borne *P. tabacina* sporangia travel long distances (Aylor et al., 1982). When these sporangia descend on a tobacco field during a rain shower or heavy dew, the potential for germination may be diminished. Nevertheless, high humidity or rainfall could change the leaf surface environment (by dilution), resulting in conditions that result in stimulation of germination rather than inhibition. The observed *in vitro* stimulatory effect on germination by the dilute DVT-diols was significant but not highly significant. In the field, however, the stimulatory influence of leaf surface chemicals on germination could mean the difference between infection and the development of an epidemic or not. The germination of one sporangium could start the disease process.

Effects of cuticular exudates on pathogens and competitive microorganisms and other leaf surface microflora have not been thoroughly investigated. Studying these interactions of microorganisms with leaf surface chemicals could be important to the future management of foliar disease.

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HOW CONTACT FORAGING EXPERIENCES AFFECT PREFERENCES FOR HOST-RELATED ODORS IN THE LARVAL PARASITOID *Cotesia marginiventris* (CRESSON) (HYMENOPTERA: BRACONIDAE)[1]

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Abstract—Responses of individual females of the parasitoid *Cotesia marginiventris* to the odors of four different complexes of host larvae feeding on leaves were observed in a four-arm olfactometer. The plant–host complexes were composed of fall armyworm (FAW) larvae or cabbage looper (CL) larvae feeding on either corn or cotton seedlings. Prior to testing, each female was given a brief foraging experience on a plant–host complex and was then exposed to the odors of the same complex in the olfactometer. The experienced females responded to familiar odors in a dose-related manner, and these responses were virtually identical to all four complexes. Preferences for the odors of one of two plant–host complexes were tested in dual choice situations. Generally, FAW odors were preferred over CL odors and corn odors over cotton odors. A short foraging experience significantly affected the females' odor preferences in favor of the odors released by the experienced complex. Additional experiments revealed that neither longer bouts of experience nor bouts that included ovipositions resulted in a stronger change in preference. Experience affected preference in combinations where only the host species was varied as well as in combinations where only the plant species was varied. The results, therefore, strongly indicate that both the plants and the hosts somehow are involved in the production and/or release of the semiochemicals that attract *C. marginiventris*.

[1] Mention of a proprietary product does not constitute an endorsement or the recommendation for its use by the USDA.

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Key Words—Hymenoptera, Braconidae, *Cotesia marginiventris*, host-finding, semiochemicals, conditioning.

INTRODUCTION

Females of many insect parasitoids rely on host and host-habitat related chemicals as cues in their search for hosts (for reviews see Vinson, 1976, 1981, 1984; Weseloh, 1981; van Alphen and Vet, 1986). Several studies have demonstrated that the response to these semiochemicals is flexible and can be influenced by learning (Thorpe and Jones, 1937; Monteith, 1963; Arthur, 1971; Taylor, 1974; Vinson et al., 1977; Sandlan, 1980; Strand and Vinson, 1982; Vet, 1983, 1988; Vet and van Opzeeland, 1984, 1985; Wardle and Borden, 1985; Dmoch et al., 1985; Drost et al., 1986; Lewis and Tumlinson, 1988). These studies show that experiences with hosts and/or their microhabitats, both by immature and mature stages, may influence an adult parasitoid's response to semiochemicals. *Cotesia marginiventris* (Cresson), a solitary larval parasitoid of many Lepidoptera, shows a significant increase in response to host-related odors after only a brief contact experience with host damaged leaves contaminated with host by-products (Turlings et al., 1989). After females receive a contact experience with a particular plant–host complex, they respond significantly better when they are exposed to the odors of that plant–host complex than when they are exposed to the odors of an alternative plant–host complex (Turlings et al., 1989). This suggests that the experience effect is not merely the result of a general increase in response to semiochemicals, but that the insects actually learn to respond to the odors that they encounter during their experience.

This phenomenon of conditioning through experience has been suggested as a useful method for biological control programs in which parasitoids could be stimulated to respond to host-related odors prior to their release in a target area (e.g., Lewis and Nordlund, 1985). This would be particularly helpful if the wasps would not just increase their responses to the experienced odors, but would actually prefer these odors over those released by alternative plant–host complexes when given a choice.

Here we report on a study in which the effect of experience on odor preferences by *C. marginiventris* was tested. Shifts in preference in favor of an experienced odor were studied in situations where only the host species was varied and in situations where only the plant was varied. Thus, we obtained information on the specific roles played by both host larvae and plants in the production and release of semiochemicals essential for host-habitat location.

METHODS AND MATERIALS

Population of C. marginiventris. Parasitoids of the '85 Mississippi strain were reared on fall armyworm larvae at the USDA-ARS, Insect Biology and Population Management Research Laboratory, Tifton, Georgia, according to the procedure described by Lewis and Burton (1970) for the parasitoid *Microplitis croceipes*. Cocoons were collected a few days prior to emergence and shipped to Gainesville where they were kept in 25 × 25 × 25-cm Plexiglas cages, each with one side of fine mesh nylon screen. Parasitoids that emerged on the same day were kept in the same cage, and all cages were stored in cabinets at 26°C, 50–60% relative humidity, and a 15-hr photophase. Males were removed after two days, allowing sufficient time for all females to be mated. All experiments were conducted with 3- to 5-day-old mated females, 6–10 hr into the photophase.

Hosts. The hosts used in the experiments were second-instar larvae of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), and of the cabbage looper (CL), *Trichoplusia ni* (Hübner). They were reared according to the procedure described by King and Leppla (1981).

Olfactometer. Individual females were exposed to host-related odors and observed in a four-arm olfactometer similar to the one described by Vet et al. (1983) with some modifications described by Eller et al. (1988). This device is designed so that four well-defined odor fields, each associated with one of the arms, are created in a square central arena. The fraction of an odor-containing flow that actually reached a parasitoid in the olfactometer could be controlled by diverting part of the flow before it entered the arena. The fraction that was split off was then replaced by clean humidified air. The total flow entering the central exposure chamber through each arm was kept at 300 ml/min.

Odor Sources. The odor sources consisted of five late second- or early third-instar larvae feeding on three seedlings. The larvae of either FAW or CL were put on the seedlings of either corn (*Zea mays* L.) or cotton (*Gossypium hirsutum* L.) 1.5 hr prior to the actual bioassays.

Data Recording. The behavior of the females in the olfactometer was recorded with the use of an Epson Geneva PX-8 portable computer. After a female was introduced into the olfactometer, the time it spent in each odor quadrant was recorded during a 5-min period. If the parasitoid walked into one of the arms and did not return within 15 sec, this was recorded as a final choice for that arm. The remaining time was added to the time spent in the quadrant of the final choice. For the dual choice tests, the odor quadrant in which a female spent the greatest amount of time was recorded as her odor field preference.

RESULTS

Dose–Response Experiments. Before actual odor preferences were tested, the responses to different odor doses were tested for each plant–host complex separately. Thus, dose–response curves could be generated to determine which concentrations of the different plant–host complexes evoke similiar response levels. These concentrations were then used in the preference experiments to reduce the possibility that preferences were influenced by concentration differences.

The responses of female parasitoids were observed to the odors of FAW on corn, FAW on cotton, CL on corn, or CL on cotton. During each test the odor of one of these complexes was offered through one of the four flows, while the other flows contained humidified air and served as controls. For each of the four odor sources three concentrations were tested: 25, 50, and 100% of the original odor flow.

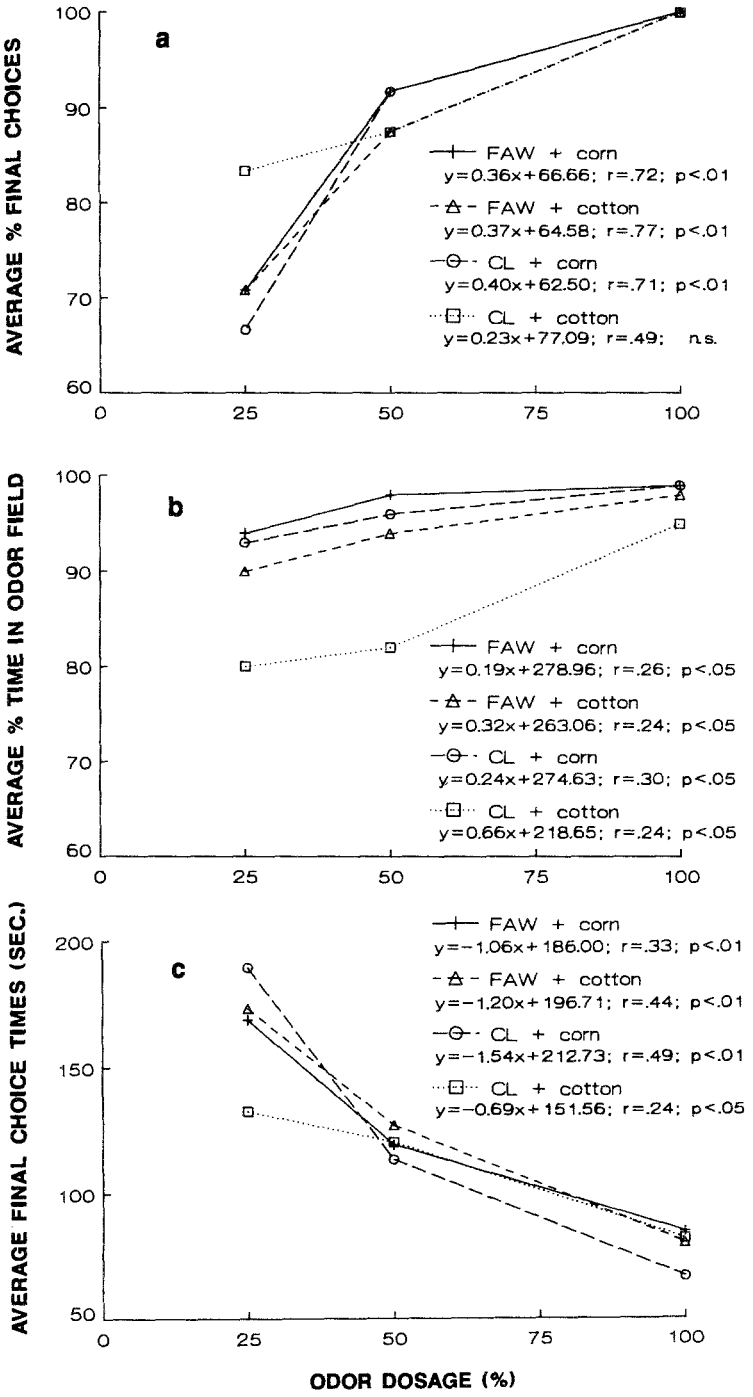
Just prior to being tested, a female was placed, for 20 sec, on a plant–host complex like the one used as the odor source. The parasitoid was prevented from actually encountering hosts. This type of experience significantly increases a female's response to host-related odors (Turlings et al., 1989). The female was then introduced into the olfactometer. Odor sources were rotated to the next air flow after six females were tested for each of the three concentrations of a particular odor. A total of 24 females were tested for each concentration of all odor sources.

Figure 1 shows that the wasps responded in a dose-related manner to all four complexes. Regression analyses of the time spent in the odor quadrant (Figure 1b) and of the time it took the females to make a final choice (Figure 1c) show a significant increase in responsiveness to the odors with increasing odor dose. The total number of final choices made for the odor arms was also found to be significantly dose related, with the exception of CL on cotton (Figure 1a).

No significant differences were found between the same doses of the four odor sources. In all of the following preference tests, 50% doses were used.

Effect of Short Contact Experience on Odor Preference. The effect of a short-term contact experience on a female's preference for the odors emitted by

FIG. 1. Responses of experienced *C. marginiventris* females to three doses of odors emitted by larvae feeding on leaves. Responses were measured as: (a) average percentage of the females that made a final choice for the odor arm; (b) average percentage of time that the females spend in the quadrant with the odor; (c) average time it took a female to make a final choice. The lines connect the average values, while the equations for the actual linear regressions are given with each graph.


a
AVERAGE % FINAL CHOICES
100
90
80
70
60
0
25
50
75
100
FAW + corn
y=0.36x+66.66; r=.72; p<.01
FAW + cotton
y=0.37x+64.58; r=.77; p<.01
CL + corn
y=0.40x+62.50; r=.71; p<.01
CL + cotton
y=0.23x+77.09; r=.49; n.s.
b
AVERAGE % TIME IN ODOR FIELD
100
90
80
70
60
0
25
50
75
100
FAW + corn
y=0.19x+278.96; r=.26; p<.05
FAW + cotton
y=0.32x+263.06; r=.24; p<.05
CL + corn
y=0.24x+274.63; r=.30; p<.05
CL + cotton
y=0.66x+218.65; r=.24; p<.05
c
AVERAGE FINAL CHOICE TIMES (SEC.)
200
150
100
50
0
25
50
75
100
FAW + corn
y=−1.06x+186.00; r=.33; p<.01
FAW + cotton
y=−1.20x+196.71; r=.44; p<.01
CL + corn
y=−1.54x+212.73; r=.49; p<.01
CL + cotton
y=−0.69x+151.56; r=.24; p<.05
ODOR DOSAGE (%)

different plant–host complexes was tested in a series of dual-choice experiments. A female wasp was allowed to contact, for 20 sec, a plant–host complex from which the larvae had been removed. Immediately following this contact experience, the wasp was transferred to the olfactometer. In the olfactometer, the wasp was exposed to the odors of two different plant–host complexes introduced through adjacent arms of the olfactometer; the two remaining arms carried humidified air only. One of the odors was from the complex that the female had just encountered, the other odor from a different complex. One combination of two odor sources was tested on a given day with 8–10 females that experienced one source and the same number of females that experienced the other source, before being introduced into the oflactometer. This was replicated six times for each combination.

The results, as summarized in Figure 2, show that the probability that a female chooses the odor of a particular complex is higher if she has had experience with that complex than if she has had experience with the other complex. The T test (SAS Institute, 1987) was used to make an overall comparison of the responses to the odors of complexes with which females had experience to those with which they had no experience. The differences were highly significant for both the number of final choices ($N = 6$; $T = 4.17$; $P = 0.009$) and the odor field preference ($N = 6$; $T = 4.69$; $P = 0.005$). However, for each individual combination, the differences in odor preferences were not always significant (Figure 2). For the numbers of final choices made for the odors, a significant difference was found only for the combinations FAW on corn versus CL on corn ($\chi^2 df_1 = 8.624$; $P = 0.003$) (I, Figure 2c) and CL on corn versus CL on cotton ($\chi^2 df_1 = 3.907$; $P = 0.048$) (IV, Figure 2c). Odor field preference was affected significantly for the combinations FAW on corn versus CL on corn ($\chi^2 df_1 = 6.596$; $P = 0.010$) (I, Figure 2d) and FAW on corn versus CL on cotton ($\chi^2 df_1 = 4.318$; $P = 0.038$) (V, figure 2d).

Pooling the combinations with different hosts feeding on the same plants (I and II in Figure 2) and the combinations with the same hosts feeding on different plants (III and IV) revealed an overall preference for FAW and for corn odors. The females divided their final choices more or less equally among FAW and CL (73:62), but the overall odor field preference deviated significantly from a 1:1 ratio (116:76; $\chi^2 df_1 = 3.802$, $P = 0.051$). Plant preference in favor of corn was demonstrated with both the number of final choices (98:45; $\chi^2 df_1 = 9.778$, $P = 0.002$) and the odor field preference (131:68; $\chi^2 df_1 = 9.285$, $P = 0.002$).

Effects of experience were also analyzed with a loglinear model (SAS Institute, 1987) with five dependent variables: experienced host, experienced plant, alternative host, alternative plant, and preference (experienced or alternative

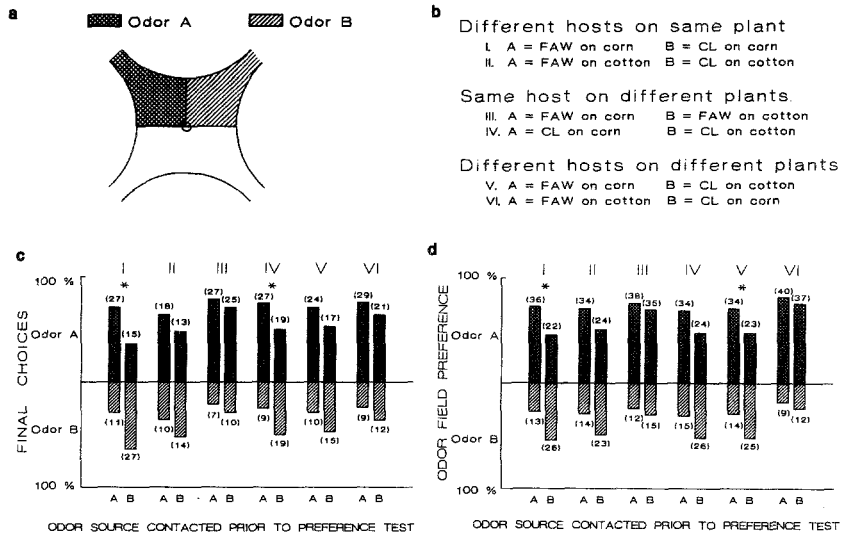


FIG. 2. Effects of experience on the preference of *C. marginiventris* females for host-related odors. (a) Diagram of the olfactometer with the odors of two different plant–host complexes entering the exposure chamber through adjacent arms. (b) List of the six plant–host complexes that were tested. (c) Summary of results using the percentage of females that made a final choice for a specific odor arm as the measure of response. Females that did not make a final choice were excluded. (d) Summary of results using the percentage of females that spend most of their time in a specific odor field (= odor field preference) as the measure of response. The few females that never entered one of the two odor fields were excluded. The roman numerals (I–VI) in (c) and (d) refer to the combinations listed in (b). In (c) and (d) the bars above the x-axis represent the females that choose odor A and below the x axis the females that choose odor B. The actual numbers are shown in parentheses. For each combination, 48–50 females were tested of both experience types. The asterisks indicate significant differences in total numbers due to experience (chi-square; $P < 0.05$).

odor). The number of females responding on a test day was used as one observation. There were 24 response levels with a total frequency (N) of 415 for the final choices and 585 for the odor field preference. Again, the overall effect of experience in favor of the experiences odor was highly significant [final choices (FC): χ^2 df_1 = 12.21, P = 0.0005; odor field preference (OFP): χ^2 df_1 = 13.84, P = 0.0002]. Significantly more females chose the experienced odor if they had experienced corn than if they had experienced cotton (FC: χ^2 df_1 = 15.82, P = 0.0001; OFP: χ^2 df_1 = 12.55, P = 0.0004). No such difference in experience effect was found for the two host species. The general preference

for FAW odors was demonstrated by the fact that significantly more females would choose the alternative odor if the alternative host was FAW (FC: χ^2 df_1 = 10.32, P = 0.0013; OFP: χ^2 df_1 = 19.85, $P < 0.0001$). No such difference was found for the plant species. Note that in those cases where the wasps experienced the source with the least preferred host and plant (i.e., CL on cotton; combinations II, IV, and V) no preference for either odor source was observed.

The combination FAW on corn–CL on cotton was chosen for the following additional preference experiments. This combination contains all four components, and the results obtained for this combination allow room for measurable increases and decreases in the effect of experience.

A More Complete Experience. The previous experiments were conducted with females that had a 20-sec contact experience without ovipositions just prior to their introduction into the olfactometer. Further experiments were performed to determine whether a longer, more complete experience, which included ovipositions, would result in a stronger effect upon the odor preference by the parasitoids, and whether this effect of experience would be retained over time.

Females were experienced by placing them in a glass container (26 cm diam., 10 cm high) containing either 70 FAW larvae on 12 corn seedlings or 70 CL larvae on 12 cotton seedlings. The containers were then covered with a Plexiglas plate. All females made contact with the plants and frass, and parasitized more than one larva. Females were exposed to the plant–host complex until they left the plants and attempted to leave the container. The exposure time varied from 4 to 11 min.

The persistence of the experience effect over time was tested by giving one group of females their experience in the morning 3–4 hr before being tested in the olfactometer (group 1); a second group was given their experience just a few minutes prior to the bioassay (group 2).

Results for the two groups are presented in Figure 3. Again, the differences between females that experienced different complexes were slight but consistent. The preference for the odor of FAW on corn was less for the females that experienced CL on cotton. For the females of group 1, the difference in preference was only found to be significant for the number of final choices (χ^2 df_1 = 5.326; P = 0.021). For group 2, a significant difference between females experienced on a different complex was only found in the odor field preference (χ^2 df_1 = 15; P = 0.001).

Group 1 and group 2 females did not differ from each other in their response to the odors except for females experienced on FAW on corn. Group 2 females with a FAW on corn experience preferred FAW on corn odors significantly more than group 1 females that had experienced the same complex (χ^2 df_1 = 5.87; P = 0.015). This difference was not observed in the number of final choices made by the two groups.

Experiences with Ovipositions versus Experiences without Ovipositions.

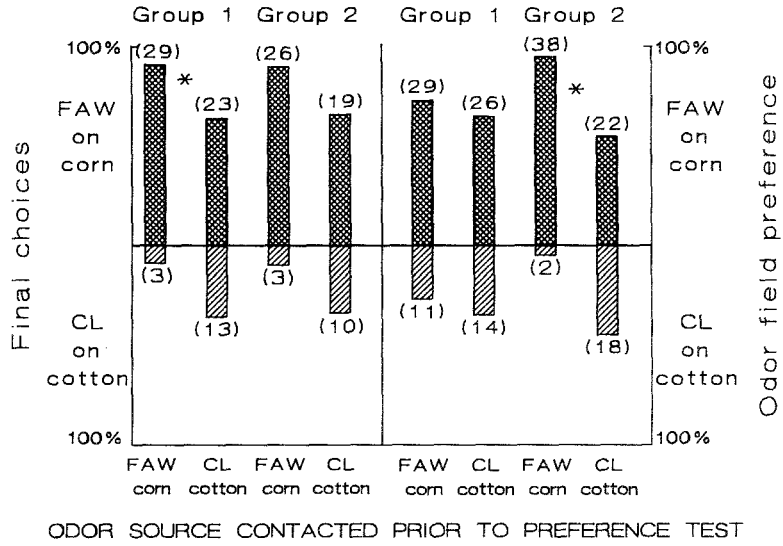


FIG. 3. Responses of *C. Marginiventris* females to the odors of either of two plant-host complexes after a complete contact experience including ovipositions as indicated in the figures. Group 1 had their experience 3–4 hr prior to a bioassay; group 2 had their experience just prior to a bioassay. The actual numbers are shown in parentheses. The asterisks indicate significant preference shifts.

Finally, treatments were tested simultaneously to reveal possible differences between experiences with ovipositions and experiences without ovipositions, which may so far have been hidden by interday variation. Two sets of females were given a complete experience as described above, 1 hr before the bioassays. The first set encountered larvae and could oviposit freely during the experience. The second set of females, however, was experienced on a complex where the larvae were removed so that only the contaminated and damaged leaves could be contacted.

The results were very similar to those found for the treatments discussed before (Figure 4). No differences were found between females that had a total experience including ovipositions and females that only contacted the damaged and contaminated leaves.

DISCUSSION

The dose–response tests revealed that the females' responses increase with an increasing dose of the host-related odors. No significant differences were found in the attractiveness of the four different plant–host complexes when the

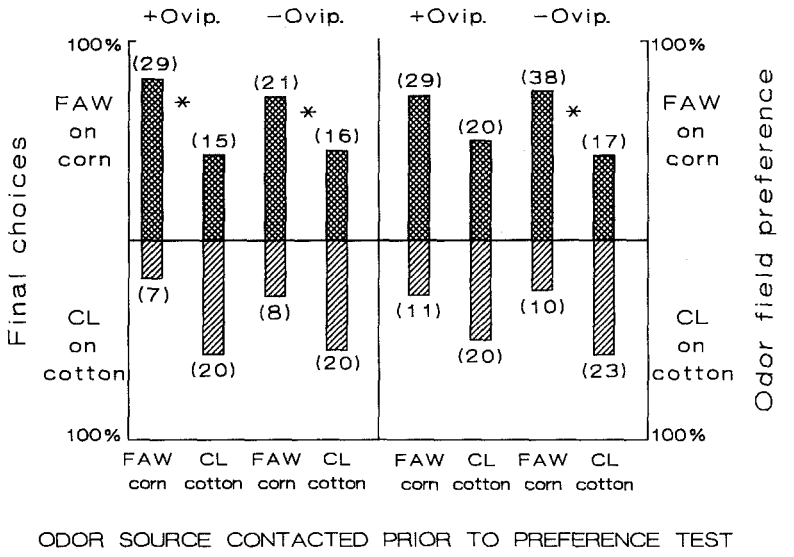


FIG. 4. Responses of *C. marginiventris* females to the odors of two plant-host complexes after a complete contact experience with (+Ovip.) and without (−Ovip.) ovipositions. The actual numbers are shown in parentheses. The asterisks indicate significant preference shifts.

parasitoids were exposed to the odors in single choice tests. The pooled results of the preference experiments, however, indicate a preference for FAW odors over CL odors and an even stronger preference for corn odors over cotton odors. Since FAW seems to be *C. marginiventris'* most important host and FAW larvae are found predominantly feeding on corn, other legumes, and grasses (Ashley, 1986), an innate preference for the odors of FAW larvae and damaged corn is not surprising. Furthermore, CL appears to be a very poor host since initial rearing experiments show minimal emergence from this host (unpublished data; M.R. Strand, personal communication). On the other hand, since all test animals were reared on FAW larvae, the observed preference for FAW may also have been the result of conditioning of the parasitoids as immatures. However, since host larvae are routinely fed artificial diet, the rearing procedure could not account for the corn preference.

The results not only indicated an innate preference for FAW and corn odors, they also showed that the preferences were affected by contact experiences with the plant-host complexes. For all plant-host complex combinations, it was

found that a particular complex was chosen more often by females that had experienced that complex than by the females that had experienced the alternative complex. Although not always significantly, this experience effect did cause a change in preference in each individual combination. The overall effect was found to be highly significant.

The results are in agreement with earlier results of single choice experiments presented by Turlings et al. (1989). The increase in response to host-related odors after experience is greatest to the odors emitted by the plant–host complex that the females experienced. The learning process that must be involved is triggered by a brief contact with host by-products and does not require actual contact with the hosts. When females were given a longer experience period, including ovipositions, they did not appear to respond differently than females that had a 20-sec experience without ovipositions. The effect of experience on the preference for host-related odors lasted at least several hours and is therefore likely to be an important factor determining the host-searching behavior of these parasitoids in the field.

Significant differences in preference were found when females were offered odor source combinations where only the host species varied, where only the plant species varied, and where both the host and the plant varied. We can therefore conclude that the females are able to distinguish between different host species and between different plant species. Evidently, both host and plant are somehow involved in the emission of the semiochemicals that evoke a response in the parasitoid females, either by producing the essential volatiles or by affecting the volatiles released by another component of the complex. The parasitoids are therefore likely to respond to more than one compound, and the intensity of their response to each compound probably increases when it is encountered in association with a foraging experience. The results suggest that each plant–host complex releases its own blend of semiochemicals that is detected by *C. marginiventris*. After exposure to a particular complex, a female will subsequently be attracted to an odor blend that is most similar to the blend she perceived during her experience. Future research will have to reveal whether females of *C. marginiventris* and other parasitoid species distinguish between variations in specific semiochemical blends or whether they are able to differentiate between different compounds altogether.

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EUROPEAN CORN BORER SEX PHEROMONE
Inhibition and Elicitation of Behavioral Response by Analogs

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Abstract—The male sexual behavior-stimulating and inhibiting properties of a series of analogs of the European corn borer sex pheromone were determined in a flight tunnel. The structural requirements for inhibition of pheromonal response were far less restrictive than those for elicitation of that response. Analogs that by themselves elicited upwind flight response from males at a low dose were generally less inhibitory to male response than many of the analogs that had no pheromonal activity. These findings suggest that many pheromone analogs bind to pheromone receptors without provoking behavioral response and possibly undergo slower degradation on the antenna than pheromonally active compounds. The disparity of response to analogs by two pheromonal types of the European corn borer indicates that the pheromone receptor and pheromone catabolic systems are biochemically very different in the two types.

Key Words—*Ostrinia nubilalis*, Lepidoptera, Pyralidae, European corn borer, (*E*)- and (*Z*)-11-tetradecen-1-ol acetate, pheromone analogs, insect behavior, sex pheromone, inhibition.

INTRODUCTION

The male European corn borer (ECB), *Ostrinia nubilalis*, offers an excellent opportunity for the study of the mechanisms involved in pheromone perception because of the simple composition of its female sex pheromone, a mixture of the geometrical isomers of 11-tetradecen-1-ol acetate. Furthermore, the ECB occurs in three genetically distinct pheromonal types that use different propor-

tions of the geometrical isomers (Klun and Maini, 1979) and affords the possibility of carrying out intraspecific studies.

In our recent investigation (Schwarz et al., 1989) of structure–activity relationships of the ECB pheromone, modifications were made in the alkyl moiety of the pheromone molecule. It was found that requirements for the elicitation of male behavioral response in a flight tunnel by analogs were extremely restrictive and that compounds that acted as good pheromone mimics in the flight tunnel assay were only marginally effective in field trapping tests. Remarkably, while the most active pheromone mimic in the flight tunnel assay, i.e., (*Z*)-12-cyclopropyl-11-dodecen-1-ol acetate, had potent pheromonal activity against the Z-type ECB, the corresponding *trans*-isomer was pheromonally inactive against the E-type ECB. Furthermore, the *trans*-isomer was also found to inhibit the response of the E-type ECB to its natural pheromone when it was coevaporated at a high dose with the pheromone. This result indicated that assays for pheromonal activity per se may not be the only approach to define spatial and/or functional group requirements for interaction with the pheromone chemoreceptor system and that inhibition of biological response to pheromone by analogs may be equally informative in furthering our understanding of the phenomena involved in pheromone perception.

In this paper, we report the pheromonal and inhibitory activities of a series of analogs of 11-tetradecen-1-ol acetate and indications of a structure–activity relationship for these behavioral manifestations in two pheromonal types of the ECB.

METHODS AND MATERIALS

Insects. ECB males used in the laboratory bioassays were from colonies that were homozygous for Z- or E-female sex pheromone production genes (Klun and Huettel, 1988). The Z-type utilized 97:3 (*Z*/*E*)-11-tetradecen-1-ol acetate as its pheromone, E-type used 3:97 *Z*/*E*. Inasmuch as the ECB is nocturnal with regards to its mating activities, the colonies were maintained in environmental chambers under a reversed photoperiod [80% relative humidity (RH); 16 hr light–8 hr dark, 26°C–20°C] so that bioassays could be conducted during working hours.

Flight-Tunnel Bioassays. The flight tunnel used in all bioassays was the same as described by Raina et al. (1986). Conditions in the tunnel were: 18–20°C, 40–60% RH, 2.5 lux (red light), and a wind speed (laminar flow) of 50 cm/sec. In assays for pheromonal activity, 100 ng of analog alone was used as the stimulus. Assays were replicated ($N = 15$ or 20) and the percentage upwind flight response to each analog was normalized relative to response to natural pheromone (100%). In one case (see Table 4 below), males were exposed to

different doses of analogs along with 100 ng of pheromone and the mean percentage upwind flight response at each dose was calculated. In inhibition assays 3 μg of analog was presented to males together with 100 ng of pheromone. In some tests, males were preexposed to 3 μg of analog for 3 min and then exposed to 3 μg of analog plus 100 ng of pheromone. All compounds were applied to a 0.5 $\times$ 4-cm filter paper strip in 3–5 μl heptane and the strip was positioned on a clip that was suspended on a vertical wire 16 cm from the tunnel floor and 20 cm from the upwind screen-covered end of the tunnel. Males were positioned in a release tube that was 300 cm downwind of the stimulus. When the lid of the release tube was removed or when a screen was used for preexposure to analog, the stimulus plume passed directly through the tube. Adult males, 2–3 days old, were used in the bioassays after they had been held in the flight tunnel room for ca. 30 min at 2.5 lux and 20°C. Males were tested individually against a treatment and then discarded. Tests were conducted during hours 5–6 of scotophase.

All inhibition assays were conducted using a complete block design with replication over time. Each male tested was allowed 3 min to respond after the lid or the screen of the release tube was removed. In all assays, *cis*-analogs were tested against Z-type ECB and *trans*-analogs were tested against E-type ECB and in some instances reciprocal assays were conducted. As control in every test, one treatment always consisted of natural pheromone displayed alone.

Males used in each test were divided into separate populations; one for each treatment in a test. In each population, four categories of response were evaluated: no response, partial upwind flight (male flew upwind in plume but a distance less than 298 cm), upwind (flight in stimulus plume from release tube to within 2 cm of the source of the stimulus), and land (landing on filter paper strip and display of precopulatory behavior). The four categories were assigned response values of 0, 1.4, 2.8, and 3, respectively.

The following description of the statistical methods is for inhibition tests having three treatments but tests with two or four treatments were handled similarly. The number of insects in each response category was assumed to follow an independent multinomial distribution for each population. The SAS Categorial Modeling Procedure (SAS Institute, 1988) was used to analyze the data. The linear model, $D_i = M + t_i$ ($i = 1, 2, 3$), was fit to the data using weighted least squares estimation. The response function, D_i, was the average score of the insects in the population, M was the overall mean score for all populations, and t_i was the treatment effect. It was assumed that $t_1 + t_2 + t_3 = 0$. If there were three response values of zero in a particular population, then the zero frequencies were replaced with the number 0.0001. This was done, for computational purposes, to indicate that the zeros were sampling zeros and not structural zeros (missing data). The first hypothesis tested was that treatment effects were equal ($t_1 = t_2 = t_3$). If this hypothesis was rejected, then the

hypotheses ($t_1 = t_2$, $t_1 = t_3$, and $t_2 = t_3$) were tested. Wald test statistics, which follow the chi-square distribution asymptotically, were used to test the various hypotheses. Because of the large number of hypothesis tests conducted and because of the effect of the moderately small ($N = 15$ or 20) population sizes for the response function used on the asymptotic chi-square tests, an effort was made to be conservative in not rejecting hypotheses. Accordingly, a significance level of $\alpha = 0.0001$ was used for all hypothesis tests. This allowed for 100 independent hypothesis tests to be performed; each at the 0.01 level [$(1 - 0.0001)^{100} \simeq 1 - 0.01$]. For the presentation of data in Tables 1–3, the mean response score, D_i, was transformed to percentage inhibition; % inhibition = $(1 - D_i/3) \times 100$ and an asterisk was used to designate statistically significant levels of inhibition relative to male response to natural pheromone alone.

Chemicals. Compound **1** was prepared by a Wittig reaction of methylenetriphenylphosphorane and 11-acetoxyundecanal and purified by preparative high-performance liquid chromatography (HPLC) on two 25-cm × 10-mm-ID stainless-steel tubes in series packed with 10 μm Spherisorb–20% $AgNO_3$ (w/w), and using toluene as eluant. Compound **1** served as starting material for **6,** which in turn was converted by standard methods into compounds **2–5** and **12.** Compound **9** was prepared from commercially available 3-dodecyn-1-ol according to the procedure of Macaulay (1980) and served as starting material for **7** and **8.** Compounds **10, 11,** and **13–15** were commercial products. Synthesis of compounds **16** and **17** has been reported (Klun et al., 1980), as was the synthesis of **18** and **19** (Schwarz et al., 1983), of **25, 26, 32, 33,** and **39** (Schwarz et al., 1986), and of **20** and **40** (Schwarz et al., 1989). Compounds **34** and **41** were prepared as a mixture of geometrical isomers by the Wittig reaction of 3-fluoropropylidenetriphenylphosphorane and 11-acetoxyundecanal. The pure isomers were obtained by preparative $AgNO_3$–HPLC. Esters **21, 24, 31, 34–36,** and **42–46** were prepared from commercially available alcohol precursors by standard methods. All new compounds had infrared, nuclear magnetic resonance, and mass spectra consistent with the proposed structures.

RESULTS AND DISCUSSION

Activity of 12-Carbon Analogs. None of the 12-carbon analogs had any pheromonal activity. As was reported previously (Schwarz et al., 1989), the degree of modification that is tolerated at the terminal ethyl group is very small. It is therefore not surprising that compound **1** (Table 1) showed no pheromonal activity. Other analogs, which deviated even more from the natural pheromone structure, were also behaviorally inactive. However, as Table 1 shows, some of these analogs displayed considerable inhibitory activity toward male upwind flight to pheromone. One can surmise that the origin of inhibitory activity lies

TABLE 1. INHIBITION OF UPWIND FLIGHT RESPONSE OF EUROPEAN CORN BORER MALE TYPES IN FLIGHT TUNNEL BY SIMULTANEOUS EXPOSURE TO 12-CARBON ANALOGS AND NATURAL PHEROMONE[a,b]

No.	Compound	Inhibition of upwind flight (%)[c]	
		E-type	Z-type
1	11-dodecen-1-ol acetate	88*	74*
2	11-dodecen-1-ol formate	32	34
3	11-dodecen-1-ol fluoroacetate	61*	22
4	11-dodecen-1-ol difluoroacetate	58*	18
5	11-dodecen-1-ol trifluoroacetate	40	14
6	11-dodecen-1-ol	100*	100*
7	11-dodecyn-1-ol acetate	90*	90*
8	11-dodecyn-1-ol formate	11	19
9	11-dodecyn-1-ol	13	36
10	dodecan-1-ol acetate	80*	24
11	dodecan-1-ol	21	18
12	11-dodecenal	59*	95*

[a] Pheromone tested against the E-type ECB was 11-tetradecen-1-ol acetate (*E*/*Z*, 97:3), and against the Z-type was 11-tetradecen-1-ol acetate (*Z*/*E*, 97:3). 3 μg analog + 100 ng pheromone were used in each test.

[b] 3 μg Analog + 100 ng pheromone were used in each test.

[c] *Denotes statistically significant level of inhibition relative to male response to natural pheromone alone.

in the ability of a compound to bind with the receptor site and thus block access of the pheromone to that site. However, because of its structural deficiency, it is unable to transmit the proper neurological signal to evoke a behavioral output.

The generally accepted picture of pheromone binding (Liljefors et al., 1985) is thought to involve a three-pronged interaction of the phereomone with the acceptor by hydrogen bonding to and/or electrostatic interaction with the functional group, electrostatic interaction with the double bond, and much weaker dispersion forces are responsible for binding the terminal alkyl group. If this picture holds true, one would expect that 11-dodecen-1-ol acetate (**1**), which is the closest structural relative of the natural pheromone, might be able to bind reasonably well to the pheromone receptor. It would therefore compete efficiently, when at high concentrations, with the pheromone and inhibit behavioral output, which was indeed the case. However, we also found (Table 1) that 11-dodecen-1-ol (**6**) was qualitatively a better inhibitor than **1**, while dodecan-1-ol acetate (**10**) showed high inhibition for the E-type ECB only. Also, 11-dodecyn-1-ol acetate (**7**) was as active in the inhibition bioassay as was **1**, while 11-dodecyn-1-ol (**9**) was practically inactive. Modification of the ester moiety

to formate (**2**), mono- (**3**), di- (**4**), or trifluoroacetate (**5**) led to compounds that are much less inhibitory, even though changes in size from **1** are minor. 11-Dodecenal (**12**), whose structure is even further removed from the natural pheromone, showed high inhibitory activity. These results illustrate the difficulty of arriving at structure–activity correlations in this series of compounds.

From these observations, it becomes evident that the simple picture of a "lock-and-key" fit of pheromone or pheromonal analog and receptor molecule, which has been applied successfully to explain steric requirements for behavioral output, does not hold for these inhibitors. Indeed, the picture is even more complicated considering that in numerous instances inhibitory activity in the E-type ECB was not mirrored by equivalent activity in the Z-type; this suggests that profound differences exist between the receptor molecules in the two ECB types.

In tests where males were preexposed for 3 min to 3 μg of analog and immediately exposed to the same dose of analog plus 100 ng of pheromone, inhibition of the upwind response was not statistically different from the level of inhibition seen when the analog was coevaporated with pheromone without preexposure to analog (27 of the analogs were tested in this mode, but data are not presented). These results indicate that a 3-min preexposure to a 3 μg dose did not cause total sensory adaptation. Tests ($N = 10$) also showed that a 3-min preexposure of males to microgram amounts of 11-dodecen-1-ol acetate (**1**) or 11-dodecen-1-ol (**6**) did not suppress immediate subsequent response to 100 ng of pheromone by itself. Furthermore, exposure of E-type and Z-type males to 3.1 μg of their respective pheromone elicited an upwind flight response that was not significantly different from the response to 100 ng of pheromone, indicating that only minor adaptation or overloading of the sensory system takes place under these conditions. These examples attest to the efficiency with which the olfactory system clears itself when exposed to physiological overdoses of olfactory stimuli. In reciprocal tests where E-type males were exposed to *cis*-isomer (**38**) and vice versa, upwind flight was always significantly inhibited. This inhibition can be accounted for by the fact that males perceive an improper geometrical isomer ratio.

Activity of 14-Carbon Analogs. Table 2 lists the inhibitory activity of the 14-carbon analogs that had no pheromonal activity. This lack of pheromonal activity strengthens our argument for the necessity of very high stereochemical mimicry to elicit behavioral activity. In general the unsaturated acetates were inhibitory while the saturated analog, tetradecan-1-ol acetate (**13**), was not. Thus, for compounds **14, 15,** and **16,** the presence of a double bond, albeit not in the 11-position, is required for inhibitory activity.

In a previous study Klun et al. (1979) showed that (*E*)-9-tetradecen-1-ol acetate (**14**) inhibited Z-type male ECB precopulatory behavior as well as attraction in field traps. More recently, Struble et al. (1987) reported that (*Z*)-

TABLE 2. INHIBITION OF UPWIND FLIGHT RESPONSE OF EUROPEAN CORN BORER MALE TYPES IN FLIGHT TUNNEL BY SIMULTANEOUS EXPOSURE TO PHEROMONALLY INACTIVE 14-CARBON ANALOGS AND NATURAL PHEROMONE[a]

No.	Compound	Inhibition of upwind flight (%)[b] E-type	Z-type
13	tetradecan-1-ol acetate	38	19
14	(*E*)-9-tetradecen-1-ol acetate	100*	NT
15	(*Z*)-9-tetradecen-1-ol acetate	NT[c]	88*
16	(*E*)-12-tetradecen-1-ol acetate	85*	NT
17	(*Z*)-12-tetradecen-1-ol acetate	NT	30
18	(*E*)-11,13-tetradecadien-1-ol acetate	88*	100*
19	(*Z*)-11,13-tetradecadien-1-ol acetate	81*	21
20	(*E*)-12-cyclopropyl-11-dodecen-1-ol acetate	87*	88*
21	(*E*)-11-tetradecen-1-ol trifluoroacetate	88*	NT
22	(*E*)-11-tetradecen-1-ol	40	NT
23	(*Z*)-11-tetradecen-1-ol	NT	83*
24	(*E*)-11-tetradecen-1-ol propionate	11	NT
25	(*E*)-13,13-dimethyl-11-tetradecen-1-ol acetate	6	NT
26	(*Z*)-13,13-dimethyl-11-tetradecen-1-ol acetate	NT	62*
27	(*E*)-11-tetradecenal	95*	NT
28	(*Z*)-11-tetradecenal	NT	100*
29	tetradecanal	50*	68*

[a] Pheromone tested against the E-type ECB was 11-tetradecen-1-ol acetate (*E*/*Z*, 97:3), and against the Z-type was 11-tetradecen-1-ol acetate (*Z*/*E*, 97:3). 3 μg analog + 100 ng pheromone were used in each test.

[b] * denotes statistically significant level of inhibition relative to male response to natural pheromone alone.

[c] NT = not tested.

9-tetradecen-1-ol acetate (**15**) also significantly reduced captures of Z-type ECB males in field trials. Glover et al. (1989) confirmed the inhibitory properties of **14** and **15** in flight-tunnel assays in both pheromonal types and found that **15** was the more inhibitory compound in either type.

Acetates **16** and **17** are pheromonal components of the Asian corn borer, *O. furnacalis* (Klun et al., 1980), which occurs sympatrically with the ECB in some regions of China (Chiang et al., 1982). Surprisingly, only the *trans*-isomer (**16**) was inhibitory in the E-type ECB, while the *cis*-isomer (**17**) had no significant activity against the Z-type.

(*E*)- and (*Z*)-11,13-tetradecadien-1-ol acetate (**18** and **19**), were tested for inhibitory activity against both pheromonal ECB types. While *trans*-analog (**18**) was an inhibitor of both ECB types, the *cis*-analog (**19**) was active in the

E-type only, again indicating the dissimilarity of steric requirements for inhibitory activity in both types of ECB.

(*E*)-11-Tetradecen-1-ol trifluoroacetate (**21**) was strongly inhibitory, while the *cis*-isomer (**44**) was less so and displayed a modicum of pheromonal activity (see below). It is interesting to note that Camps et al. (1988) found that substitution of the acetate moiety by trifluoroacetate in the pheromone of the processionary moth, *Thaumetopoea pityocampa*, resulted in complete loss of pheromonal activity and in reduction of trap catches when the analog was added to the natural pheromone.

Dissimilarities of inhibitory activity were encountered with the natural pheromone-derived alcohols **22** and **23**; (*E*)-11-tetradecen-1-ol was a much less potent inhibitor than was its *cis*-isomer. (*E*)-13,13-Dimethyl-11-tetradecen-1-ol acetate (**25**) had no inhibitory activity, while the *cis*-isomer (**26**) was inhibitory.

All aldehydes tested proved to be effective inhibitors; tetradecanal (**29**) and 11-dodecenal (**12**) inhibited both ECB types, while (*E*)-11-tetradecenal (**27**) and (*Z*)-11-tetradecenal (**28**) were potent inhibitors of the E- and Z-type ECB, respectively. One can speculate that this inhibition could arise from reversible Schiff base formation between the aldehyde moiety of the inhibitor and the receptor molecule in a manner similar to the one proposed for the olfactory receptors of the tiger salamander, *Ambystoma tigrinum*, (Mason and Morton, 1984).

Table 3 lists the analogs that were pheromonally active and their inhibitory activity. Inspection of the pheromonal activity data confirm our previous findings (Schwarz et al., 1989) that to preserve that activity only very minor deviations from the geometry of the natural structure are allowed. Thus, 11-tetradecyn-1-ol acetate (**31**), in which a triple bond is substituted for a double bond, had moderate activity against the E-type ECB and was weakly active against the Z-type; presumably the triple bond mimics the *trans*-double bond better than the *cis*-double bond. Other successful substitutions of a triple bond for a *trans*-double bond have been reported by Eidmann et al. (1986) and by Brückner et al. (1988) for the pheromones of *Ips typographus* and *Lobesia botrana*, respectively. Similarly, 13,13-dimethyl-11-tetradecyn-1-ol acetate (**33**) was weakly active against the E-type ECB and not at all against the Z-type.

Small changes in the ester moiety led to disparate results. Thus, while (*Z*)-11-tetradecen-1-ol propionate (**46**) was pheromonally very active in the Z-type, its *trans*-isomer (**24**) was completely inactive in the E-type; however, (*E*)- and (*Z*)-11-tetradecen-1-ol formate (**37** and **45**) had activities of the same order of magnitude, 63% and 47%, respectively, in both types. No structure–activity relationship can be drawn for the pheromonal activities of the mono-, di-, and trifluoroacetates of (*Z*)-11-tetradecen-1-ol (**42, 43,** and **44**). All three compounds had moderate to weak pheromonal activity in the Z-type. In the E-type

TABLE 3. UPWIND FLIGHT RESPONSE OF EUROPEAN CORN BORER MALE TYPES TO ANALOGS AND INHIBITION OF UPWIND FLIGHT RESPONSE BY SIMULTANEOUS EXPOSURE TO ANALOGS AND NATURAL PHEROMONE[a]

No.	Compound	$\bar{X}$ % upwind flight response	Inhibition of upwind flight (%)[c]	
		E-type	E-type	Z-type
30	(*E*)-11-tetradecen-1-ol acetate	100	26	72*
31	11-tetradecyn-1-ol acetate	27	98*	94*
32	(*E*)-13-methyl-11-tetradecen-1-ol acetate	65	32	52
33	13,13-dimethyl-11-tetradecyn-1-ol acetate	20	6	18
34	(*E*)-14-fluoro-11-tetradecen-1-ol acetate	100	93*	77*
35	(*E*)-11-tetradecen-1-ol fluoroacetate	79	33	NT
36	(*E*)-11-tetradecen-1-ol difluoroacetate	14	35	NT
37	(*E*)-11-tetradecen-1-ol formate	63	52	NT
		Z-type	Z-type	E-type
38	(*Z*)-11-tetradecen-1-ol acetate	100	30	90*
31	11-tetradecyn-1-ol acetate	10	94*	98*
39	(*Z*)-13-methyl-11-tetradecen-1-ol acetate	65	55	21
40	(*Z*)-12-cyclopropyl-11-dodecen-1-ol acetate	62	13	21
41	(*Z*)-14-fluoro-11-tetradecen-1-ol acetate	100	54	77*
42	(*Z*)-11-tetradecen-1-ol fluoroacetate	25	68*	NT
43	(*Z*)-11-tetradecen-1-ol difluoroacetate	8	46	NT
44	(*Z*)-11-tetradecen-1-ol trifluoroacetate	18	59*	NT
45	(*Z*)-11-tetradecen-1-ol formate	47	76*	NT
46	(*Z*)-11-tetradecen-1-ol propionate	88	24	NT
26	(*Z*)-13,13-dimethyl-11-tetradecen-1-ol acetate	6	62*	NT

[a] Pheromone tested against the E-type ECB was 11-tetradecen-1-ol acetate (*E*/*Z*, 97:3), and against the Z-type was 11-tetradecen-1-ol acetate (*Z*/*E*, 97:3). 100 ng analog was used in upwind flight response tests; 3 μg analog + 100 ng pheromone were used in inhibition assays.

[b] * denotes statistically significant level of inhibition relative to male response to natural pheromone alone

[c] NT = not tested.

the monofluoroacetate (**35**) was highly active, the (*E*)-difluoroacetate (**36**) was only weakly active, and the (*E*)-trifluoroacetate (**21**) (Table 2) was completely inactive. If pheromonal activity of the fluoro-analogs were strictly related to steric factors, one would expect all of them to be active considering the closeness in size of the Van der Waals radii of the fluorine atom and the hydrogen atom, 1.60 and 1.50 Å, (Allinger, 1976) respectively. The absence of correlation of pheromonal activity must therefore be related to electronic factors. In view of this, the pheromonal activity of (*E*)- and (*Z*)-14-fluoro-11-tetradecen-

1-ol acetate (**34** and **41**) is of considerable interest. These compounds had pheromonal activity in our flight-tunnel bioassay and in field-trapping tests (unpublished results) that was indistinguishable from the natural pheromone. This result is different from the findings of Camps et al. (1988). They found that substitution of a vinylic hydrogen by fluorine in the pheromone of the processionary moth led to an analog that was only "slightly" pheromonally active in field-trapping tests. Inhibitory effects were observed for mixtures of this analog and the pheromone.

Considering the inhibitory activity of pheromonally active analogs (Table 3), no straightforward structure–activity relationship can be discerned. However, comparison of inhibitory activity and pheromonal activity of the acetylenic and *trans*-analogs against the E-type ECB, reveal an interesting relationship. Five compounds, including the major component of the natural E-type pheromone (**30**), had pheromonal activity of 60% or higher and, of these five compounds, four had statistically nonsignificant inhibitory activity. Only (*E*)-14-fluoro-11-tetradecen-1-ol acetate (**34**) had both high pheromonal and high inhibitory activity. The same comparison of the two measured activities for *cis*-analogs against the Z-type ECB shows that there were five compounds, again including the major component of the natural Z-type pheromone (**38**), with high pheromonal activity, and all five had statistically nonsignificant levels of inhibitory activity.

The fact that we found high pheromonal activity generally to be paired with low inhibitory activity was surprising. A priori, one would have expected the natural pheromone or pheromone-mimicking compounds, when present in hyperphysiological concentrations, to overload the receptor system and therefore seemingly act as an inhibitor. Indeed, the inability to produce behavioral response under these conditions underlies one of several mechanisms proposed to explain disruption of pheromone communication (Minks and Cardé, 1988). However, for the ECB under our experimental conditions in the flight tunnel the pheromonally active analogs were not inhibitory. Thus, analogs, except one, with high pheromonal activity at a dosage of 100 ng, did not significantly inhibit upwind flight at exposure rates of 3 μg of analog plus 100 ng of pheromone. In contrast, many of the analogs that produced low or no behavioral activity at 100 ng were inhibitory when released at a dosage of 3 μg plus 100 ng of pheromone. The apparent inverse relationship between pheromonal and inhibitory activity might be explained as follows.

Perception of a steady flow of pheromone molecules, transduction to neural signals, and ultimately to behavioral output, requires not only highly specific pheromone receptors, but also specific catabolic enzymes (Vogt, 1987). It is reasonable to assume that the high specificity of pheromones and pheromone receptors is coupled to a highly specific enzymatic pheromone clearing system.

For instance, Prestwich et al. (1989) have shown in the case of the pheromone of the gypsy moth that its rate of hydration by male antennal enzymes was significantly higher for natural (+)-disparlure than for the pheromonally inactive enantiomer and was twice the rate of hydration of a pheromonally inactive analog, *meso*-11,12-epoxydicosane. Prestwich et al. (1986) also found that analogs that were conformationally similar to the acetate component of the *Antheraea polyphemus* pheromone were better competitive inhibitors of sensillar esterases than analogs that had less conformational similarity to the pheromone component. If the specificity of pheromones and pheromone receptors was indeed coupled to specificity of the pheromone clearing enzyme system, it would be expected that analogs that successfully mimic the pheromone should serve as better substrates for the pheromone-specific catabolic systems and be degraded more effectively than those compounds that are structurally less similar to the pheromone. Thus, compounds lacking sufficient pheromone mimicry would be cleared from the receptor less effectively and act as behavioral inhibitors.

In many cases, the obverse is also true. For instance, 11-tetradecyn-1-ol acetate (**31**), which had low pheromonal activity in both E- and Z-type ECB, was a very potent inhibitor of upwind flight.

It should be noted that, as a rule, the steric requirements for an analog to be a pheromone-mimicking substance are much more stringent than for the analog to be an inhibitor of behavioral output. An analog may bind to the receptor site, but this binding may not be appropriate for the transmission of a pheromonal signal. Because the analog also lacks the specificity of structure necessary for efficient interaction with the catabolic enzymes, the receptor site is unable to detect competing pheromone molecules. Preiss and Priesner (1988) studied behavioral responses of male codling moths in a flight tunnel to its pheromone and analogs, and their findings support our interpretation. They found that two analogs of codlemone were pheromonally very active at a dosage of 100 ng. When 1-μg amounts of each of these compounds was coevaporated with 1 ng of codlemone, the analog having the highest pheromonal activity had the least inhibitory effect on male response, and the analogs that were the most inhibitory had negligible pheromonal activity.

It is reasonable to assume that these pheromone-receptor sites and analog (i.e., inhibitor)–receptor site interactions are subject to equilibria governed by binding constants between pheromone or analog and receptor and that the former would be favored. As is shown in Table 4, this was the case for two of our potent inhibitors: 11-dodecen-1-ol acetate (**1**) and (*E*)-11,13-tetradecadien-1-ol acetate (**18**). Inhibition fell off rapidly when the dosage of inhibitor was reduced while the dosage of pheromone was kept constant, allowing the pheromone to better compete for interaction with the pheromone receptor.

In summary, we have shown that for the ECB the stereochemical require-

TABLE 4. INHIBITION OF UPWIND FLIGHT RESPONSE OF EUROPEAN CORN BORER MALE TYPES TO SIMULTANEOUS EXPOSURE TO VARYING DOSES OF ANALOG AND 100 ng NATURAL PHEROMONE[a] (N = 15)

No.	Amount/compound	$\bar{X}$% inhibition of upwind flight response	
		E-type	Z-type
1	3000 ng 11-dodecen-1-ol acetate + 100 ng pheromone	92	67
1	300 ng 11-dodecen-1-ol acetate + 100 ng pheromone	69	33
1	30 ng 11-dodecen-1-ol acetate + 100 ng pheromone	35	17
	100 ng pheromone	0	0
18	3000 ng *E*-11,13-tetradecadien-1-ol acetate + 100 ng pheromone	85	83
18	300 ng *E*-11,13-tetradecadien-1-ol acetate + 100 ng pheromone	92	35
18	30 ng *E*-11,13-tetradecadien-1-ol acetate + 100 ng pheromone	36	17
	100 ng pheromone	0	0

[a] Pheromone tested against the E-type ECB was 11-tetradecen-1-ol acetate (*E*/*Z*, 97:3) and against the Z-type was 11-tetradecen-1-ol acetate (*Z*/*E*, 97:3).

ments for compounds to function as inhibitors of pheromonal activity are much less stringent than for compounds to act as pheromone mimics. This, ostensibly, is due to the considerable latitude available for chemicals to bind to the pheromone receptor and block that site without undergoing rapid degradation by pheromone-specific antennal enzymes. However, for a compound to act as a pheromone mimic, a close analogy to the natural pheromone structure is required. Concomitantly, such compounds are recognized and processed efficiently by specific catabolic enzymes on the antennae. This relationship is also evidenced by our finding that pheromonally active substances generally act as poor behavioral inhibitors.

The geometrical opposition of structures found in the pheromones of the E- and Z-types of the ECB is not mirrored in the biological properties of the analogs investigated, pointing to profound biochemical differences in the receptor and catabolic systems of the two types of moths.

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TRICHOTHECENE MYCOTOXINS PRODUCED BY *Fusarium sporotrichioides* DAOM 197255 AND THEIR EFFECTS ON SPRUCE BUDWORM, *Choristoneura fumiferana*

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Abstract—Trichothecene mycotoxins were produced by *Fusarium sporotrichioides* DAOM 197255 isolated from a spruce budworm cadaver. An extract from the culture filtrate containing these metabolites was toxic to budworm when ingested at concentrations as low as 10 ppm in diet, and survivors were predisposed to infection with a fungal entomopathogen. The possible role of these metabolites in the balsam fir–spruce budworm habitat is discussed.

Key Words—Trichothecenes, sesquiterpines, *Fusarium sporotrichioides*, fungal toxins, insecticidal activity, fungal pathogen, *Paecilomyces farinosus*, spruce budworm, *Choristoneura fumiferana*, Lepidoptera, Tortricidae.

INTRODUCTION

Fusarium species produce many toxic metabolites from diverse biogenic origins including amino acid-derived compounds such as enniatins and butenolide, polyketides such as zearlenone, sesquiterpines (T-2 toxin and deoxynivalenol), and compounds of mixed biosynthesis such as fusarin C (Marasas et al., 1984). Toxin-producing *Fusarium* species are present in many substrates and habitats and can have detrimental effects on other organisms (Ueno, 1977; Drysdale, 1984).

In their study of fungi on the surface of balsam fir, *Abies balsamea* L.

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(Mill.), needles, Miller et al. (1985) reported the presence of toxin-producing fungi on foliage infested with spruce budworm, *Choristoneura fumiferana* Clem. It was later demonstrated that one of these fungi, *Fusarium avenaceum* (Fr.) Sacc, produced, among other things, enniatins that were toxic to spruce budworm (Strongman et al., 1988). Strunz and Strongman (1988) have discussed the possible effects of fungal toxins in the budworm habitat in terms of delayed larval development and susceptibility to pathogens.

Isolations of fungi from spruce budworm cadavers on balsam fir yielded *Fusarium sporotrichioides* Sherb. (DAOM 197255) (Strongman, unpublished data). This species is known to produce an array of mycotoxins including trichothecenes (e.g., T-2 toxin) (Marasas et al., 1984). Trichothecenes have been shown to be toxic to insects (Grove and Hosken, 1975) and have previously been reported as products of entomopathogenic species of *Fusarium* (Claydon and Grove, 1984). This paper concerns toxin production by *F. sporotrichioides* DAOM 197255 and its effects on spruce budworm larvae as part of the effort to determine the role of fungi in the spruce budworm-balsam fir habitat.

METHODS AND MATERIALS

F. sporotrichioides DAOM 197255 was isolated (on 2% malt extract agar) from dead budworm collected at the Acadia Forest Experimental Station, Fredericton, NB, and identified according to Nelson et al. (1983).

The fungus was grown in culture to test for toxic effects on budworm and to determine the metabolites produced. For insect testing the fungus was grown by producing a seed culture as described by Miller and Blackwell (1986) and then inoculating this (5% v/v) into Glaxo bottles containing 1 liter of a medium composed of glucose (17 g), yeast extract (4.25 g) and peptone (4.25 g) in 1 liter of distilled water filtered through a Barnstead nanopure system II. The cultures were incubated at 28°C for 28 days in the dark. At the end of this period the mycelium was separated from the medium by filtration, the culture filtrate extracted with three portions of chloroform, and then reduced to dryness (yield of amorphous extract ca. 500 mg). This extract was incorporated into spruce budworm diet at concentrations of 10, 40, 200, and 400 ppm and bioassayed for effects on growth rate and mortality as described in Strongman et al. (1988). Survivors from the culture filtrate bioassay (10 ppm) and half of the control group (0 ppm) were challenged with the pathogenic fungus *Paecilomyces farinosus* (Dicks. ex Fr.) Brown et Smith by dipping larvae in a spore suspension (1 million spores/ml). The rest of the control group were dipped in sterile distilled water. All the larvae were incubated (25°C, 18 hr light) on moist filter paper in Petri dishes to which some diet was added. Mortality was recorded after 36 and 96 hr. The cadavers were examined microscopically and cultured on 2% malt extract agar.

To determine the toxic metabolites produced by *F. sporotrichioides* DAOM 197255, a two-stage fermentation in 250-ml shake flasks was used (Miller and Blackwell, 1986). Culture filtrate (100 ml) from the production stage fermentation was put on a ClinElute column and washed with 8× 100-ml portions of ethyl acetate. The eluate was reduced to dryness. Gas chromatography–mass spectronomy (GC-MS) analysis used a Finnigan model 4500 GC-MS system with an Incos data base. Underivatized samples in ethyl acetate were injected on the column at ambient temperature. Separation was achieved on a DB-5 fused silica column (20m × 0.32 mm ID, 0.25 μm film), temperature programmed from 140 to 260°C at 15°C/min and with helium as carrier gas at 10 psi. Mass spectral data were obtained in the electron impact mode and compared with the extended *Fusarium* compound library (Plant Research Centre, Ottawa, Ontario).

To determine if *F. sporotrichioides* could produce toxins in budworm cadavers, a "medium" was prepared by homogenizing budworm (14 g frozen larvae) in 50 ml distilled water. This medium was autoclaved then inoculated with 2.5 ml of a suspension made by homogenizing an agar slant of the fungus in 50 ml of sterile distilled water. This culture was incubated at 28°C for 28 days; a dichloromethane extract was prepared and analyzed for toxins using GC-MS as described above.

RESULTS AND DISCUSSION

F. sporotrichioides DAOM 197255 from spruce budworm produced toxic metabolites characteristic of Canadian strains of this species including T-2, HT-2, diacetoxyscirpenol, neosolaniol, propyl-neosolaniol, butyl-neosolaniol and α-13-dihydroxy-apotrichothecene (Greenhalgh et al., 1988). The crude extract (500 mg) from the culture filtrate assayed for toxicity against budworm contained mainly T-2 (44.6 mg), HT-2 (181.3 mg), and neosolaniol (73.0 mg), the remainder being fatty acids, triglycerides, and ergosterol. This extract was toxic to budworm larvae at concentrations as low as 10 ppm in the diet and at 40 ppm caused ca. 75% mortality and virtually halted development (Table 1). Larvae fed extract (10 ppm) and then challenged with *P. farinosus* showed higher mortality compared to controls not dosed with toxins (Table 2). Although the sample size in the latter experiment dictates caution in drawing conclusions, the results suggest a synergism between toxin ingestion and infection with a fungal pathogen.

T-2 toxin was detected in the budworm "medium" inoculated with *F. sporotrichioides* DAOM 197255; therefore it is possible that toxins from this fungus occur in the fir–budworm habitat. This fungus was isolated exclusively from budworm cadavers in the habitat, but it is not considered an insect pathogen by Claydon and Grove (1984). It has been postulated that toxin production

TABLE 1. BIOASSAY OF CULTURE FILTRATE EXTRACT FROM *Fusarium sporotrichioides* DAOM 197255[a]

Extract (ppm)	N	Survival (%)	No. survived to each instar			
			III	IV	V	VI
0	46	91.3	6	19	17	0
10	22	77.2	13	4	0	0
40	22	27.3	6	0	0	0
200	21	4.8	1	0	0	0
400	23	0.0	0	0	0	0

[a] Second-instar spruce budworm larvae in vials with artificial diet (Strongman et al., 1988). Regression analysis showed a strong correlation ($r = 0.988$) between increased dose and mortality and differences in survival were significant by ANOVA ($P = <0.05$).

TABLE 2. MORTALITY OF SPRUCE BUDWORM LARVAE FED EXTRACT (10 ppm) OF CULTURE FILTRATE FROM *Fusarium sporotrichioides* DAOM 197255 AND INOCULATED WITH *Paecilomyces farinosus*[a]

	N	No. dead after inoculation			
		36 hr	96 hr	Total	No. with fungus
Control	5	1	0	1	0
Control inoculated	10	2	1	3	3
Treated inoculated	9	0	6	6	5

[a] Larvae were survivors of culture filtrate bioassay (Table 1).

by grain-inhabiting *Fusaria* protects the fungus from predation by insect herbivores (Dowd et al., 1989). Likewise, toxin production by this fungus may help it compete for substrate (dead budworm), but the toxins may have other effects in the fir–budworm habitat, such as synergism with other mortality factors.

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PHEROMONE VARIATION AMONG EASTERN EUROPEAN AND A WESTERN ASIAN POPULATION OF THE TURNIP MOTH *Agrotis segetum*[1]

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Abstract—The female sex pheromone composition and the male electrophysiological response with respect to the three main sex pheromone components, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate, and (Z)-9-tetradecenyl acetate, were investigated in populations of *Agrotis segetum* from Armenia and Bulgaria. The percentage composition of the female-produced pheromone was 1:52:47 and 1:42:57 for the respective populations. Corresponding male receptor frequencies were 9:90:1 and 6:92:2. EAG response profiles of the male antennae were similar for the two populations. The populations from Armenia and Bulgaria differed from the earlier investigated French and Swedish populations, which have larger amounts of (Z)-5-decenyl acetate in gland extracts and have a majority of (Z)-5-decenyl acetate-sensitive receptors. Investigation of receptor frequencies on antennae of male Hungarian moths showed that individuals could be classified as either Swedish or Armenian/Bulgarian type. Males of the Swedish type were preferentially attracted to the three-component pheromone blend, whereas blends of (Z)-7-dodecenyl and (Z)-9-tetradecenyl acetate, and (Z)-7-dodecenol [pure

[1] Schiff. (Lepidoptera: Noctuidae).

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or in mixture with (Z)-5-decenol] attracted the Armenian/Bulgarian type. The nature of pheromone variation among European and Asian populations of the turnip moth and possible mechanisms maintaining the variation are discussed.

Key Words—Sex pheromone, *Agrotis segetum*, Lepidoptera, Noctuidae, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, population variation, olfactory receptors, single sensillum response, gas chromatography, field trapping.

INTRODUCTION

Moth sex pheromones are species-specific communication systems for mate location. Theoretically the sex communication system in a species should be under strong stabilizing selection as long as the species inhabits a continuous area with similar selection pressures on the communication system throughout, due to the great reduction in mating success that an aberrant individual would experience. However, significant intraspecific variation in the pheromone composition has been documented in, for instance, the larch budmoth *Zeiraphera diniana* (Guerin et al., 1984; Priesner, 1979), the European corn borer *Ostrinia nubilalis* (Anglade et al., 1984), and the turnip moth *Agrotis segetum* (Arn et al., 1983, Löfstedt et al., 1986).

The sex pheromone communication system of *Agrotis segetum* has been studied extensively during the last decade. The main pheromone components have been identified as (Z)-5-decenyl, (Z)-7-dodecenyl, and (Z)-9-tetradecenyl acetate (Z5–10 : OAc, Z7–12 : OAc, and Z9–14 : OAc) (Bestmann et al., 1978; Arn et al., 1980; Tóth et al., 1980; Löfstedt et al., 1982). These three substances have also been shown to be detected by olfactory receptor cells situated in three physiologically distinct types of sensilla (Löfstedt et al., 1982; Van Der Pers and Löfstedt, 1986; Löfstedt et al., 1986).

Studies on a Swedish population of turnip moths showed a female pheromone production of Z5–10 : OAc, Z7–12 : OAc, and Z9–14 : OAc in a 4 : 52 : 44 ratio and male pheromone-sensitive sensilla responding to the three components in the ratio 66 : 33 : 1 (Löfstedt et al., 1986). When the Swedish population was compared to a French and a British, the French population was significantly different showing a 47 : 40 : 13 relationship between the produced pheromone components and a 87 : 12 : 1 ratio between the sensillum types. The British insects did not differ significantly from the Swedish (Löfstedt et al., 1986). French males could, in field tests, be attracted to pure Z5–10 : OAc (Arn et al., 1983), which was impossible with the Swedish insects (Löfstedt and Löfqvist, unpublished). Analysis of female pheromone production as well as analysis of male electrophysiological and behavioral response to pheromone components

has thus far demonstrated the existence of at least two different pheromone types among European turnip moths, hereafter referred to as the Swedish and the French types.

In field-trapping experiments performed in Bulgaria the differences between European populations of the turnip moth were demonstrated once more by Subchev et al. (1986), who showed that in this area male turnip moths were attracted to pure Z7–12 : OAc or Z9–14 : OAc. These two substances, mixed at a 1 : 4 ratio, also provided the best bait for the Bulgarian population.

The differences in sex pheromone composition among different geographical populations of *A. segetum* discovered in these studies suggested the present investigation to test the hypothesis that the relative amount of Z7–12 : OAc and Z9–14 : OAc in the female sex pheromone and the number of Z7–12 : OAc and Z9–14 : OAc sensitive sensilla on the male antenna are larger in individuals originating from populations from the east of Europe, compared to individuals from populations of more western or northern origin. Turnip moths from Hungary, Bulgaria, and the Soviet Republic of Armenia were collected, and the female sex phereomone composition and the ratio of different physiological sensillum types on the male antenna were determined.

METHODS AND MATERIALS

Insects. Larvae of *A. segetum* were collected from alfalfa fields near Yerevan, Armenia. Bulgarian and Hungarian moths were supplied from cultures established from eggs of feral females caught by light traps. The larvae were reared on a semisynthetic diet (Nagy, 1970) at 28°C and an 18-hr/6-hr light–dark cycle. The pupae were sexed, and the males were shipped to the Swedish laboratory for electrophysiological single sensillum recordings or their electroantennographic response profiles were investigated in the Hungarian laboratory. Males used for electrophysiological experiments were from the second and third laboratory generations. The females were allowed to emerge in Hungary. Two days after emergence, the female pheromone gland was excised and extracted in hexane, whereafter the extracts were sent to Sweden for analysis. The females extracted were from the first, second, and fourth laboratory generations. Feral males used in the study were caught in pheromone traps in Hungary (see Field Experiments) and were transported live to Sweden to be examined electrophysiologically using the single-sensillum recording technique.

Field Experiments. Hungarian *A. segetum* males for electrophysiological investigation, caught on different baits, were collected from sticky traps in Hungary.

Field tests were conducted using triangular traps made from transparent

polyethylene sheets. Baits were applied in 10 μl hexane solution to 1-cm pieces of red rubber tubing (Bora'szati Szaküzlet, Budapest). All chemicals were supplied by Dr. S. Voerman (Institute of Pesticide Research, Wageningen, The Netherlands). The trapping experiments were conducted May 27–28 and August 1–2, 1988, in Budakeszi, Pest County, Hungary.

In a first experiment the traps were baited with 500 μg Z7-12:OH. In a second experiment the traps were baited with five different lures. The first series was baited with Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc in a 1:1:1 mix; the second series was baited with Z7-12:OAc and Z9-14:OAc in a 1:1 mix; the third series was baited with Z5-10:OAc and Z7-12:OAc in a 1:1 mix. In these three series, the amount of each component was 20 μg. The fourth series was baited with Z7-12:OAc only in a very low amount (0.05 μg), and the fifth series was baited with Z5-10:OH and Z7-12:OH in a 1:1 mix in very high amounts (500 μg of each compound). The last two series were included as they had been shown to attract large numbers of males in earlier field trapping experiments in Hungary (Tóth and Szöcs, personal communication). Insects caught in the traps were gently removed from the traps, put into envelopes, and transported to Sweden, where they were investigated electrophysiologically one to two days later.

Chemical Analysis. Extracts of female pheromone glands were analyzed by gas chromatography on a HP 5880 gas chromatograph equipped with a flame-ionization detector. The sealed ampoules were opened and 6 ng of an internal standard [(Z)-8-tridecenyl acetate] in 3 μl hexane was immediately added. After concentration of the sample to approximately 5 μl at room temperature, the hexane extract was injected on a 30-m-long × 0.25-mm-ID fused silica DB-wax column (cross-linked polyethylene glycol) (J&W Scientific, Folsom, California 95630). Conditions of chromatography were: hydrogen carrier gas velocity 40 cm/sec at 80°C; injector temperature 225°C; split valve opened 1 min after injection; temperature maintained at 80°C for 2 min following injection and then programmed at 10°C/min to 230°C. The amounts of the pheromone components were calculated relative to the internal standard comparing peak heights.

The precision of the quantification was estimated by the analysis of a synthetic reference mixture on five occasions during the two days of insect analysis. Approximately 5 μl of a hexane mixture containing 0.3 ng of the internal standard per microliter and five pheromone gland constituents (see Results) were injected each time.

Electrophysiological Methods. The response profiles of Armenian and Bulgarian male *A. segetum* antennae were recorded using the electroantennographic technique (EAG) (Schneider, 1957). Whole-body preparations were used. One platinum electrode, connected to ground, was inserted into the abdomen of the insect, and one, connected to a high impedance amplifier, was inserted into the

tip end of the antenna. The responses were measured in millivolts, and, to correct for antennal fatigue, the response to a reference substance (Z5–12:OAc) was measured between stimulations with the test compounds. One microgram of a compound to be tested was applied on a piece of filter paper, which was placed in a Pasteur pipet. A puff was then distributed from the pipet into an airstream flushing over the preparation.

Single sensillum recordings were performed with the tip-recording technique (Kaissling, 1974; Van Der Pers and Den Otter, 1978). An antenna was excised from a male moth and placed in a pipet electrode filled with Beadle-Ephrussi Ringer. The electrode was connected to ground by an Ag–AgCl wire. A pheromone-sensitive olfactory sensillum trichodeum was cut using two microscopic glass knives and a second pipet electrode, with an opening of about 2 μm, was positioned over the cut surface of the sensillum. This electrode was connected to a high-impedance amplifier by an Ag–AgCl wire. The stimulus was dispensed on filter paper. One microgram of the Z5–10:OAc and 10 μg each of the other behaviorally active pheromone components (Z7–12:OAc and Z9–14:OAc) were tested. The stimulus paper was put into a 5-ml plastic disposable syringe. One milliliter of the syringe atmosphere containing the stimulus molecules was then injected into a purified and humidified airstream flushing over the antennal preparation at a speed of 0.5 m/sec. The receptor potential and the action potentials elicited in the sensillum by the stimulus were visualized on a digital storage oscilloscope and recorded on tape on a RACAL four-channel tape recorder.

To determine the relative frequency of receptor cells for an individual male, a sample of 10 sensilla, chosen randomly on the third, fourth, or fifth segment from the base of the male antenna, was examined.

RESULTS

Chemical Analysis. Analysis of the synthetic reference mixture ($N = 5$) demonstrated the precision of the gas chromatographic quantifications. The major pheromone component Z7–12:OAc amounted to 2.58 ng (coefficient of variation 2.3%). The relative amounts of the other compounds (Z7–12:OAc always 100 by definition) were: Z5–10:OAc 17.8 (coefficient of variance 1.8%); Z7–12:OH 23.8 (1.5%); Z9–14:OAc 35.2 (3.7%); Z11–16:OAc 2.4 (10.8%).

Compounds in the pheromone gland extracts (Table 1) were identified by comparing their retention times to those of synthetic standards. Based on the batch extracts ($N = 6$, each batch containing extracts from about 10 females) the Z7–12:OAc titer of an average female *A. segetum* was 0.17 ng (range 0.04–0.33) and 0.016 ng (range 0.002–0.042) for the Armenian and Bulgarian insects, respectively. The low titer of the major pheromone component did not allow

TABLE 1. RELATIVE AMOUNTS OF ACETATES AND ALCOHOL (AVERAGE AND RANGE) IN GLAND EXTRACTS FROM TWO POPULATIONS OF *Agrotis segetum*[a]

Population	Z5-10:OAc	Z7-12:OAc	Z7-12:OH	Z9-14:OAc	Z11-16:OAc
Armenian	<2.5	100	88 (65-117)	90 (69-11)	35 (25-51)
	(*N* = 3)	(*N* = 6)	(*N* = 6)	(*N* = 6)	(*N* = 6)
Bulgarian	<2.4	100	179 (51-316)	134 (85-221)	68 (34-138)
	(*N* = 2)	(*N* = 6)	(*N* = 6)	(*N* = 4)	(*N* = 4)

[a]Z7-12:OAc = 100 by definition.

precise quantification of the minor components, nor was positive identification possible for all compounds earlier reported (Löfstedt et al., 1986). The behaviorally active acetate Z9-14:OAc was abundant in both the Armenian and Bulgarian insects, whereas the relative amount of Z5-10:OAc was below 3% of the Z7-12:OAc amount in both populations. The limit of quantification was set by the baseline noise level and the titer of the major pheromone component. In those extracts of the Armenian and Bulgarian populations that contained the largest amounts of Z7-12:OAc, the relative Z5-10:OAc titers were 2.4 and 0.7 (or less), respectively. Thus the percentage composition of the pheromone gland secretions from Armenian and Bulgarian females was calculated as 1:52:47 and 1:42:57, respectively, with regard to the homologous acetates Z5-10:OAc, Z7-12:OAc, Z9-14:OAc. It should be emphasized that the Z5-10:OAc figures are maximal estimates and that the real figures might be even lower.

Electrophysiological Analysis. Of about 100 single sensilla investigated in each population, the Armenian males possessed 9% Z5-10:OAc specialized sensilla and the Bulgarian males 6% compared with the 66% of the Swedish insects and 87% of the French (Figure 1). Thus there is a significant difference in the proportion of the different sensillum types on the male antenna among the Armenian/Bulgarian and the Swedish and the French populations. The similarity between the Armenian and the Bulgarian populations was corroborated by the EAG measurements that showed identical responses from males from the two populations. Among the Hungarian laboratory-reared insects, striking differences in receptor frequencies were observed between antennae from different males. Some of the antennae showed a proportion of Z5-10:OAc sensilla similar to that of the Swedish insects and some showed a proportion similar to the Bulgarian and Armenian insects. These two categories were designated Hungarian type 1 and Hungarian type 2, respectively. A male was assigned to type 1 if it had seven or more Z5-10:OAc sensilla in the sample of 10 and was

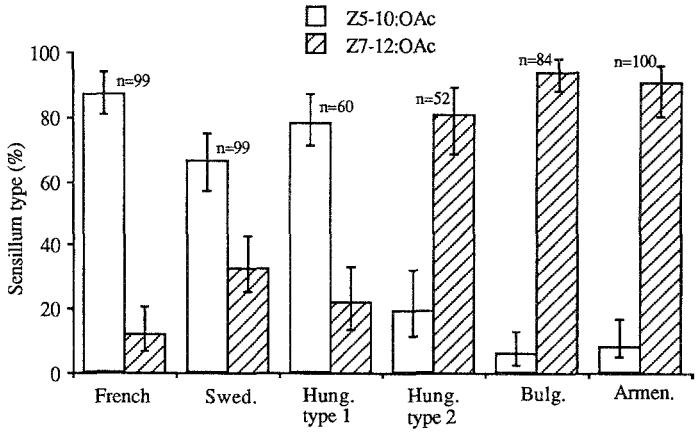

FIG. 1. Percentage of sensilla containing receptors tuned to the female sex pheromone components Z5–10:OAc and Z7–12:OAc in *Agrotis segetum* laboratory cultures of different origin, investigated in this report and in Löfstedt et al. (1986). Vertical bars indicate 95% confidence interval.

assigned to type 2 if it had three or less Z5–10:OAc sensilla in the sample. No insects fell between the groups. The difference between the most similar samples assigned to different groups was statistically significant at the 90% level ($P < 0.1$) according to the Fisher's exact probability test (Siegel, 1956). In all the investigated populations the Z9–14:OAc sensitive sensilla constituted a proportion of about 1% (range 0–2%).

To investigate the Hungarian population of the turnip moth further, males were selected by being caught in traps baited with either the three-component blend, a mixture of Z7–12:OAc and Z9–14:OAc, a mixture of Z5–10:OAc and Z7–12:OAc, pure Z7–12:OAc, or a mixture of Z5–10:OH and Z7–12:OH. The last treatment was included because in a preliminary experiment males caught in traps baited with pure Z7–12:OH, possessed only 19% Z5–10:OAc sensilla (Hungarian type 2). The males caught were typed as Hungarian type 1 or Hungarian type 2.

In the traps baited with the complete three-component mixture, both types of insects were caught, while all insects except three caught on the three different baits lacking Z5–10:OAc in their bait showed a typical type 2 sensillum frequency (Table 2). The field experiments showed clearly that both the Hungarian type 1 and type 2 of *Agrotis segetum* coexist in the field and that the type 1 males require Z5–10:OAc in the blend in order to be attracted. The type 2 males, however, seemed to be attracted to all the baits tested.

TABLE 2. TOTAL NUMBER OF ANIMALS CAUGHT IN TRAPS WITH DIFFERENT BAITS AND TYPE OF 10 RANDOMLY CHOSEN MALES FROM EACH TRAP CATCH (TYPE 1 WITH AT LEAST SEVEN Z5-10:OAc SENSITIVE SENSILLA OR TYPE 2 WITH AT MOST THREE Z5-10:OAc SENSILLA OUT OF 10 INVESTIGATED SENSILLA[a]

Bait (μg)	No. caught	Type 1	Type 2
Z5-10:OAc (20)/Z7-12:OAc (20)/ Z9-12:OAc (20)	143	6	4
Z5-10:OAc (20)/Z7-12:OAc(20)	7	2[b]	2[b]
Z7-12:OAc(20)/Z9-14:OAc (20)	46	2	8
Z7-12:OAc (0.05)	160	1	9
Z5-10:OH (500)/Z7-12:OH (500)	109	0	10

[a]The field trappings were performed May 27–28 and August 1–2, 1989, in Budakeszi, Pest county, Hungary.
[b]Out of 4.

DISCUSSION

The turnip moth is clearly polymorphic with respect to the sex pheromone communication system. The Armenian and Bulgarian insects differ so much from the French ones described earlier that an examination of only the pheromone system would probably have classed the two types as different species. The studies of different populations in this paper and in Löfstedt et al. (1986) imply that the European distribution of *A. segetum* is divided into at least three main pheromone populations: a French population producing more Z5-10:OAc and possessing many Z5-10:OAc receptors, a Swedish population producing less Z5-10:OAc and possessing fewer receptors for this compound, and an Armenian/Bulgarian population producing very little Z5-10:OAc and possessing even fewer receptors for this component. The detailed distribution of the different strains is unknown, but the large areas that the different populations occupy and the coexistence of at least the Swedish and Armenian/Bulgarian populations in Hungary suggest that we are dealing with different, more or less isolated, populations rather than with a cline.

The low pheromone titer and the large variation between batches observed in the GC analysis of the Armenian and Bulgarian gland extracts may be due to the fact that only a few of the extracted females actually contain measurable amounts of pheromone. The quantification of phereomone differences among different populations of *A. segetum* is a delicate task not only because of this but also because of the large number of pheromone component candidates involved. Taking these facts into consideration, the results obtained with dif-

ferent techniques, and by different groups of researchers in different laboratories, are remarkably coherent and consistent.

The dominance of Z7–12 : OAc and Z9–14 : OAc in extracts of Bulgarian *A. segetum* females corresponds to the importance of these two compounds for attraction of Bulgarian males, earlier reported by Subchev et al. (1986). In the case of Z9–14 : OAc we hypothesized that the importance of this compound for the Bulgarian population should be reflected in a larger number of receptors for this compound on the male antenna. This was obviously not true.

Our evidence for the cooccurrence of different *A. segetum* pheromone strains in Hungary could be further corroborated by the analysis of offspring from individual field-collected mated females. The male phenotype of the offspring can be assigned by electrophysiological means and the female phenotype by GC analysis of pheromone gland extracts, preferably from individual insects. Assortive mating under natural circumstances would result in apparent linkage between female pheromone production and male pheromone response. Different pheromone strains of the turnip moth might also coexist in other parts of Europe. Analysis of female moths of Swedish origin demonstrated a high variation in ratios between the homologous acetates among individuals (Löfstedt et al., 1985). As a matter of fact, the samples contained females, which, in hindsight, could have been classified as French type or Armenian/Bulgarian type, whereas other individuals conformed to the average Swedish type (Löfstedt et al., 1982).

The evolution of different pheromone strains, as we see in the turnip moth, requires that the stabilizing selection, which is normally assumed to operate on communication systems, must be overcome. The easiest way to envisage how the variation in a sex pheromone communication system can develop is through geographical isolation of different populations from each other (Mayr, 1963). If populations are separated during an evolutionary time span, the selection pressures acting on them in their respective areas might vary considerably and differences between the populations can build up. The interference from another, or several other species, producing a similar sex pheromone in an isolated part of the species disjunct area, would constitute a strong selective pressure for that part of the population to change its pheromone composition, as individuals would spend a lot of time and energy searching for mates of the wrong species. Another explanation could be that mutations, which influence the sex pheromone production and response, may occur in one area but not in the others and may go to fixation by pure chance (Wright, 1955).

A differentiation of the sex communication system in allopatry would make it plausible that the species populations differentiated enough, both ecologically and with respect to genetic incompatibilities, so that the differences formed in allopatry would persist also when the populations came into sympatry or parapatry. If the differentiation of the populations has proceeded so far that hybrids

produced through interpopulation matings would experience a lower fitness than the parents, the pheromone differences in the sympatric areas could become even more accentuated through reinforcement (Dobzhansky, 1940; Butlin, 1987). This reinforcement could occur even if the differentiation had not reached the level of genetic incompatibility, but remained ecological, based on sex pheromone composition differences. As hybrids in some species have been shown to exhibit an intermediate sex pheromone production and response, the hybrids might suffer a substantial loss in fitness. The only moth species that, so far, has been genetically dissected with respect to sex pheromone production and response, *Ostrinia nubilalis* (Klun and Maini, 1979; Hansson et al., 1987; Roelofs et al., 1987; Löfstedt et al., 1988), has at least three genes involved in the control of differences between strains in the sex pheromone communication. These genes are not linked (Löfstedt et al., 1988), which can make hybrid females produce one ratio and hybrid males respond to a different one. These circumstances could be involved in a reinforcement process based on communication incompatibilities.

Pheromone differences could possibly also develop in a species where the populations never became separate, that is, where they remained in sympatry or parapatry through the whole process of divergence. The populations would then be genetically compatible and continuous, but different selective pressures in different areas would create and maintain the differences between the populations (Littlejohn, 1977). The sympatric/parapatric system relying on pure ecological isolation is, however, very unstable. As soon as the selection pressure was relaxed, and insects of the aberrant populations suffered no loss in fitness by mating with an animal from the original population, the polymorphism would rapidly break down (Littlejohn, 1977). If, however, the selection pressures remained strong long enough for the differentiation of the extremes in the species distribution to proceed far enough, and maybe also become associated with some parallel changes in other ecological factors (for instance, host-plant preference), this system might also lead to a stable polymorphism.

Our evidence that two turnip moth populations coexist in Hungary under similar selection pressures on the sex communication system indicates that the populations today are truly parapatric. The two populations also seem to be fairly well defined reproductively. In traps lacking the Z5–10:OAc, almost only Hungarian type 2 males were caught, while in the traps with the three component mixture both types of males were caught. This raises the possibility that the turnip moth in Europe today is a complex of sibling species with different sex pheromones, as was recently shown in a group of primitive New Zealand tortricids (Foster et al., 1986) and for the dingy cutworm complex, *Feltia jaculifera* (Gn.) (Lepidoptera: Noctuidae) in Canada (Struble et al., 1988). The genetic differences between the populations are still to be investi-

gated to extend the picture of the pheromone system variation in *Agrotis segetum*.

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SEASONAL CHANGES OF FURANOCOUMARIN CONCENTRATIONS IN LEAVES OF *Heracleum lanatum*

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Abstract—Concentrations of three dermatitis-inducing furanocoumarins—xanthotoxin, bergapten and psoralen—were measured in whole leaves of *Heracleum lanatum* and in extracts of the leaf surface over an entire vegetative season. The concentrations of surface furanocoumarins, localized by extraction involving brief dipping in almost-boiling water followed by HPLC quantitative analysis, increased until the middle of May and decreased until maturity. The concentration on autumn leaves (new growth) was 20–100 times as high as the ones in May, or those of similar size in April. Furanocoumarin concentrations in the whole leaf at different stages of leaf development varied, being the highest April 25, then decreasing sharply with rapid leaf enlargement. Again, in the small autumn leaves the coumarin concentration was two to three times that in April. Seasonal changes in surface furanocoumarins may be important in that these compounds are postulated to form the first defense barrier of the plant.

Key Words—Furanocoumarins, *Heracleum lanatum*, leaf surface.

INTRODUCTION

The genus *Heracleum* (Umbelliferae), in common with numerous other genera of this family and of the Rutaceae in particular, is characterized by its ability to elaborate linear furanocoumarins, or psoralens (Murray et al., 1982). These compounds exhibit marked biological activity. They have been implicated in the induction of photophytodermatitis (Klaber, 1942; Musajo et al., 1954; Pathak and Fitzpatrick, 1959; Camm et al., 1976a; Evans and Smidt, 1980; Murray et al., 1982; Ceska et al., 1986) and toxicity to insect herbivores (Ber-

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enbaum, 1978, 1981; Berenbaum and Neal, 1985; Towers, 1986; Zangerl and Berenbaum, 1987; Feeny, 1987), and they can act as phytoalexins (Camm et al., 1976b), antimicrobials (Fowlks et al., 1958; Weimarck and Nilsson, 1980; Hishmal et al., 1986; Towers, 1986, 1987a), antifungals (Towers, 1987b; Chakraborty et al., 1957), insect antifeedants (Muckensturm et al., 1981), mutagens (Musajo, 1955; Cole, 1971; Clarke, 1975; Scott et al., 1976; Ashwood-Smith et al., 1980; Averbeck et al., 1987), cytotoxins (Murray et al., 1982), and plant growth regulators (Fowlks, 1953; Friedman et al., 1982; Shimomura et al., 1982; Nitao, 1988). Evidence for their involvement in skin carcinogenesis in animals (Griffin et al., 1958; Zajdela and Bisanqui, 1981) and probably in man (Stern et al., 1979; Grekin and Epstein, 1981; Ashwood-Smith et al., 1980) also exists.

Although numerous publications have dealt with isolation and identification of psoralens from many species of *Heracleum* and of their biological action, there has been little investigation of the distribution of these coumarins in tissues of the species that synthesize them and of changes in them during the course of normal development or in response to environmental influences. We have conducted such investigations with the genus *Ruta* and some other species of the above-mentioned families (Zobel and Brown, 1988a,b, 1989, 1990), but the only other relevant studies on *Heracleum* appear to be works of Carbonnier et al. These workers demonstrated quantitative variations in the content of coumarins among the various organs (Carbonnier and Molho, 1978). An examination of the fruits of several species (Carbonnier-Jarreau et al., 1978b) involving solvent extractions and UV microscopy in situ revealed that the furanocoumarins were localized exclusively in the seed integument, accumulating in the secretory canals, especially in the vittae. In another study (Carbonnier-Jarreau et al., 1978a), in which changes in metabolic patterns associated with the successive formation of dihydrofuranocoumarins and linear and angular furanocoumarins were reported, they claimed to have observed no climate-related differences in these patterns between greenhouse-grown *Heracleum* specimens and those cultivated at an altitude of 700 m.

In this paper we have studied changes, in the whole leaf and on the surface, in the concentrations and proportions of the three linear furanocoumarins most active in photosensitizing the skin—psoralen, xanthotoxin, and bergapten—in *H. lanatum*, the only native North American species, during one season's growth in a natural southern Canadian habitat.

METHODS AND MATERIALS

The 20 plants of *H. lanatum* used in these studies were growing in the wild state on the campus of Trent University. At each collection, 14–20 leaves of similar size, one from each plant, were divided into two equal samples for

analysis. Leaves were collected during the entire vegetative period of 1988, starting with the youngest leaves on April 25 and thereafter at intervals in accordance with visible changes in development, such as a rapid increase in size during May. As only green leaves were compared, the limit of the collection time was determined by the appearance of yellowish and drying leaves in early July. Two growth periods occurred in 1988: spring–summer and late November. As the small leaves grew rapidly in the spring, early samples were collected at weekly intervals. The largest leaves collected were on May 31, and thereafter mid-sized, green, healthy, undamaged mature leaves were taken on June 24 and, finally, on July 8, when, after five days with temperature maxima of 30–36°C, the leaves began to die. After autumn rains had subsequently promoted new leaf growth, additional leaves of very small size (less than half that of the first spring ones) were collected on November 22, followed a week later by others about three times as large. The first November collection was immediately preceded by two successive nights of light frost (−1°C).

Analytical procedures have been reported in earlier papers and are outlined only briefly here. The method for removal of surface deposits by a single brief dipping of the leaves into almost-boiling water has recently been described (Zobel and Brown, 1988a, 1989). Psoralens were analyzed quantitatively by high-performance liquid chromatography (Thompson and Brown, 1984; Zobel and Brown, 1988a, 1989) on a 7.5-cm reversed-phase, 4 μm C_{18} column (Waters Nova-Pak) developed with 25% aqueous acetonitrile. Quantitation was by measurement of peak areas and reference to standard curves prepared from reference samples in our collection.

RESULTS AND DISCUSSION

In the present study we determined seasonal changes in the concentrations of three furanocoumarins—psoralen, xanthotoxin, and bergapten—in the whole leaf of *H. lanatum* and on the leaf surface.

Changes in Total Coumarins. On the surface, the sum of the concentrations of these three furanocoumarins (Table 1) was, on the youngest leaves collected April 25, 0.61 μg/g fresh weight, which accounted for 0.28% of the total furanocoumarins in the leaf. After a week, on leaves by that time two to three times as large, it had dropped to 0.40 μg/g, but was still 0.33% of the total. This change paralleled the rapid leaf growth at that stage of development. The total weight of furanocoumarins per leaf increased from 285 to 375 μg.

In a week's time, by May 9, the weight of the leaves and the surface concentrations had both increased by a factor of three, amounting then to 1.2% of the total. The next stage of maturation of the leaves was followed by a slower weight increase. By May 15 the leaves were slightly larger (25% by weight),

TABLE 1. LEAF CONCENTRATIONS OF THREE PSORALENS[a] OF *Heracleum lanatum*

Date (1988)	Leaf diameter (cm)	Leaf weight (g)	Σ surface conc.[b] (μg/g)	Σ whole leaf conc.[b] (μg/g)	Percent on surface	Total in one leaf (μg)	Total on one leaf surface
4/25	5.8	1.3	0.61 ± 0.03	220 ± 9	0.28	285	0.80
5/2	9.2	3.1	0.40 ± 0.01	120 ± 4	0.33	375	1.2
5/9	17	9.5	1.25 ± 0.09	110 ± 5	1.2	1000	12
5/15	25	11.9	1.7 ± 0.10	93 ± 3	1.9	1100	20.5
5/31	43	19.6	0.87 ± 0.06	28 ± 2	3.1	570	17
6/24	28	12.9	0.72 ± 0.06	9 ± 0.5	7.2	125	9.3
7/8	27	12.3	>0.53 ± 0.04	35 ± 3	1.5	440	6.5
11/22	3.5	0.53	>32 ± 0.4	460 ± 30	6.9	260	67.5
11/30	7.1	1.80	>59 ± 2	560 ± 40	11	1100	107

[a] Psoralen + xanthotoxin + bergapten. Σ values are of the average of the replicate values determined for each of these three coumarins (see Table 2).
[b] All concentrations are expressed on a fresh weight basis.

and the increase in surface concentrations was ca. 40%, the latter value being the maximum reached in the spring–summer leaves.

After the next two weeks, by May 31, the weight per leaf increased 60%. Surface concentrations diminished over 40%; however, if this were expressed in terms of the percentage of the total concentration in the leaf, there was an increase from 1.9 to 3.1%. From that date until July, the surface concentrations on the late-spring and summer leaves decreased progressively. Again, if we compare the total concentrations of the three furanocoumarins on the surface as a percentage of the total of the entire leaf, there was a steady increase during the spring, with the maximum of 7.2% reached on June 24 followed by a diminution by July 8 to 1.5%. This could reflect loss of furanocoumarins by slow evaporation during five very warm days (maxima 30–36°C) that occurred at that time; alternatively, it may indicate that passage of furanocoumarins to the surface slowed or stopped during maturing of the leaves and the onset of senescence. We consider a loss to be more likely because, parallel to the decreasing surface concentration, the total concentration of the furanocoumarins in the whole leaf increased fourfold.

In the whole leaf the total concentration of the three furanocoumarins decreased steadily with increasing leaf weight until the June 24 sampling, a decline of >20-fold. This was followed by a sharp fourfold increase in the July 8 sample. Nevertheless, the value of 35 μg/g was still much smaller than the 220 recorded for the earliest spring leaves.

Following an abnormally warm summer, autumn rains promoted growth of new leaves. Although by November 22 these leaves were still less than half the size of the April leaves, their weight during the next week increased to 1.8 g, comparable to that of the spring ones. The surface concentration was over 50 times as high as in the April 25 leaves, and ca. 80 times as high as on those collected May 2. It was about 7% of the total in the whole leaf. By November 30 the leaves, now weighing 1.8 g, had a total surface concentration over 100 times that of the spring leaves, amounting to 11% of the total, the highest surface concentration observed. As the concentration in the whole leaf was slightly over twice as high, these data suggest that the autumn leaves exported much more of the furanocoumarins (> 100 times) to the surface than the spring–summer ones.

In an individual leaf the production of the furanocoumarins proceeded in parallel with leaf growth, because the larger leaf contained more until May 15 (Table 1, column 7). In the spring the total in one leaf increased until mid-May (hence in parallel with leaf growth), when it was 1100 μg per leaf, and then decreased to 570 in spite of the continuing increase in the leaf weight from 12 to 20 g. After four weeks, this value had again decreased to 125 (in a leaf two thirds as large), and then by the beginning of July there had been a three- to fourfold increase over a two-week period. This sudden production of furanocoumarins without any increase in leaf size again could be a normal developmental feature of the plant or a response to five days of above-normal temperatures. This phenomenon needs more investigation, including experiments with high and low temperatures under controlled conditions.

The total amount of furanocoumarins on the surface of one leaf steadily increased until mid-May, and then decreased until July 8, despite an increase in size from 11.9 to 19.6 g/leaf from May 15 to 31. The progressive decrease of the amount on the surface of one leaf is consistent with loss by slow vaporization, chemical destruction, or removal by the action of rain. In the autumn leaves, amounts on the surface (67.5–110 μg/leaf) were 10–20 times as high as in July, and > 100 times those on the small spring leaves. This increase followed the concentrations in the whole leaf, indicating a slightly higher level of synthesis accompanied by a 10–20 times greater export to the surface. Here, as in the case of the sharp increase in July discussed above, only controlled temperature experiments would appear capable of resolving the question of possible temperature effects.

Changes in Individual Coumarins. Table 2 shows that during the whole vegetative period xanthotoxin was predominant both on the surface and in the whole leaf. Its concentrations on the surface varied by a factor of 100 between spring and autumn leaves. On the spring–summer leaves they were highest in May (9th and 15th) and then decreased on fully grown leaves (31st). The high-

TABLE 2. CONCENTRATIONS OF PSORALEN, XANTHOTOXIN, AND BERGAPTEN ON SURFACE AND IN LEAVES OF *Heracleum lanatum*

Date (1988)	Surface concentrations[a]			Concentrations[a] in whole leaf			Percent on surface		
	P	X	B	P	X	B	P	X	B
4/25	0.02, 0.02	0.45, 0.495	0.13, 0.11	6.5, 6.1	150, 140	68, 64	0.32	0.32	0.18
5/2	0.02, 0.02	0.33, 0.31	0.06, 0.06	4.8, 4.6	73, 69	42, 40	0.43	0.45	0.15
5/9	0.05, 0.07	1.0, 1.1	0.19, 0.11	14, 11	65, 63	33, 25	0.48	1.6	0.52
5/15	0.035, 0.04	1.0, 1.1	0.70, 0.56	6.0, 4.8	58, 56	35, 26	0.74	1.8	2.0
5/31	0.22, 0.195	0.38, 0.44	0.27, 0.23	1.1, 1.1	28, 20	2.7, 2.5	19	1.7	9.6
6/24	0.04, 0.08	0.44, 0.52	0.18, 0.185	0.68, 0.72	6.0, 6.3	1.8, 1.95	8.6	7.7	9.6
7/8	[b]	0.40, 0.41	0.10, 0.14	1.2, 1.0	24, 20	10, 12		1.9	1.1
11/22	[b]	23, 23	9.4, 9.0	6.5, 5.7	350, 336	133, 125		6.7	7.1
11/30	[b]	34, 30	29, 25.5	5.9, 5.7	370, 350	150, 140		8.8	19

[a] All concentrations are expressed in duplicate as μg/g fresh weight.
[b] Below limit of accurate measurement.

est concentration was two to three times as high as the lowest. But on autumn leaves concentrations up to 100 times as high were observed. The concentration of xanthotoxin on the surface, taken as a percentage of the whole concentration in the leaf, was in the range 0.32–7.7% on spring–summer leaves. Values increased till June 24, and then decreased markedly by July 8. In the autumn the values were again high.

In the whole spring–summer leaves the concentration of xanthotoxin was highest at the beginning, decreasing until June 24 by a factor of ca. 30. In the leaves at the next stage of leaf development, when the plant bore yellowish and dry leaves after five very warm days, the xanthotoxin concentration in the green leaves was 3.6 times as high as two weeks earlier.

Bergapten concentrations on the surface were considerably lower than those of xanthotoxin, except for the sample of November 30, where the concentration was only slightly lower. The bergapten concentration on spring–summer leaves was highest on May 15 and then decreased steadily until July 8. The concentrations on the autumn leaves were still ca. 15–40 times as high as the highest in the spring. In the whole leaf, the bergapten concentrations were 1.9–150 μg/g during the whole period, a variation of ca. 70-fold, and less by factors of ca. 2–10 than xanthotoxin. In the spring–summer leaves the concentration differences were smaller, ca. 35-fold. Bergapten concentrations in the whole spring–summer leaves were the highest at the outset (April 25), and decreased steadily till June 24, but then rose by a factor of almost six on July 8. These tendencies were thus similar to those of xanthotoxin. In autumn leaves, the concentration was over 10 times that in summer (July 8) and over twice that of the youngest spring leaves.

If we calculate the concentrations of bergapten on the surface as a percentage of the whole leaf concentration, larger differences (0.18–19%) are observed among the samples over the whole period of study than for xanthotoxin (0.32–8.8%). Thus over the entire vegetative period the differences were >100 times, compared to only 30 times for xanthotoxin. In the spring–summer leaves, the differences for bergapten were 60 times, while for xanthotoxin they were <30 times.

This percentage value is low on the small spring leaves (0.18%), increasing with leaf maturation and then remaining steady on the mature leaves with the same drop observed for xanthotoxin on the July 8 sample. On autumn leaves the values for bergapten on the surface were 7.1 and 19%.

Psoralen concentrations on the surface were the lowest of these three furanocoumarins, varying between 0.21 and 0.02 μg/g, or even down to values below our limit of measurement. In the whole leaves also psoralen concentrations were the lowest, showing an 18-fold variation, but variations during the vegetative period were less than for the other two. Concentrations of psoralen

on the surface are very difficult to compare because of the small amounts, which were less by factors of 20–150 than that of xanthotoxin. The highest was 0.21 μg/g on the May 31 sample. In the whole leaves the highest psoralen concentration was recorded on May 9, with a steady decrease then ensuing until June 24. In the sample of old leaves collected July 8, the concentration of psoralen was almost double that of the previous one, exhibiting, on a smaller scale, the same tendency observed for xanthotoxin and bergapten.

Expressed as a percentage of those in the whole leaf, the psoralen concentrations on the surface were highest on May 31, decreasing more than one half by June 24. The values in the next three samples were below our limit of measurement.

Table 3 compares ratios of concentrations with xanthotoxin taken as unity. Although the concentrations of bergapten and psoralen were always lower, those of the individual coumarins did not exhibit the same pattern. Thus, surface bergapten decreased until May 9, then increased fourfold and remained stable for the rest of that month, decreasing afterwards until July. In the case of surface psoralen, it was stable until May 15, increased over 10 times by the end of that month, and then increased only 40% in June. The bergapten concentration in the whole leaf was steady until May 15 and then dropped by a factor of five by May 31, increasing afterwards. For psoralen the values varied more in the spring, with the peak on May 9, then decreasing in the May 31 sample, increasing again in June, with a decrease again observed July 8. We are unable to draw any inferences from such wide fluctuations.

TABLE 3. RATIOS OF CONCENTRATIONS OF PSORALEN, XANTHOTOXIN, AND BERGAPTEN ON SURFACE AND IN WHOLE LEAF OF *Heracleum lanatum*

Date (1988)	Ratio on surface			Ratio in whole leaf		
	p	X	B	p	X	B
4/25	0.04	1	0.26	0.043	1	0.45
5/2	0.06	1	0.19	0.058	1	0.57
5/9	0.06	1	0.14	0.19	1	0.46
5/15	0.04	1	0.60	0.10	1	0.54
5/31	0.51	1	0.61	0.037	1	0.10
6/24	0.73	1	0.38	0.11	1	0.30
7/8	[a]	1	0.29	0.05	1	0.50
11/22	[a]	1	0.40	0.19	1	0.38
11/30	[a]	1	0.86	0.018	1	0.36

[a] Below limit of accurate measurement.

CONCLUSIONS

In this work we have proved not only that furanocoumarins exist on the surface of *H. lanatum* (Zobel and Brown, 1988b), but also that the surface concentrations and those of the whole leaf varied throughout the vegetative period. This must have ecological significance. Our finding that, after cold nights in the autumn, there was a drastic increase both in the whole leaves and on the surface, is in agreement with a report (Beier and Oertli, 1983) that various kinds of stress—exposure to low temperature, UV irradiation, and sodium hypochlorite—lead to increased formation of furanocoumarins. Another such stress condition is infection. In celery infected with *Sclerotinium* (pink rot fungus), furanocoumarin concentrations 25 times normal have been recorded (Ashwood-Smith et al., 1985), and over 30 times during infection with *Erwinia* (Surico et al., 1987).

From the ecological point of view, the proportions of the three psoralens can be important as well, since synergism, for example, between myristicin and xanthotoxin (Berenbaum and Neal, 1985), is known to occur. We believe that the actual situation over the surface of *Heracleum lanatum* leaves may be more complex than would be produced by the slow evaporation of psoralens alone into the surrounding atmosphere, because in the extracts from the surface there are certainly more as yet unidentified components (Zobel and Brown, unpublished). The layer above the cuticle is said by Baker (1982) to contain waxes and an undefined group of compounds designated as "phenolics." Wollenweber and Dietz (1981) identified over 400 different flavonoids deposited in the leaf wax, leaf resin, and bud exudate of various plants. Furanocoumarins localized on the surface have been suggested as insect attractants (Ashwood-Smith et al., 1983; Städler and Buser, 1984). We postulate that they also have a basic role in the plant's defense system and that, if UV activation of these compounds (Towers and Yamamoto, 1985) is considered, their localization on the surface has been a logical evolutionary development.

Although trichomes have been described by Kelsey et al. (1984) as the first line of a plant's defense, our concept of a detectable concentration of vaporized furanocoumarins in the atmosphere immediately surrounding the plant (Zobel and Brown, 1989a, 1990) would imply a further extension of this barrier. In addition to being distasteful (Harborne, 1985), such compounds may also be repellently malodorous to herbivores.

Our observation that there is a large absolute decrease in the levels of psoralens in the whole leaf during part of the vegetative period (Table 1) is a clear indication that these compounds undergo further metabolism in the plant, in this species at least, and are therefore not end products. Although there has been some evidence that such is the case (Murray et al., 1982), the question

has not been extensively studied, and the nature of the metabolic products remains unknown.

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CUTICULAR HYDROCARBONS OF FOUR POPULATIONS OF *Coptotermes formosanus* SHIRAKI[1] IN THE UNITED STATES
Similarities and Origins of Introductions

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Abstract—The degree of similarity among cuticular hydrocarbon profiles of four populations of *Coptotermes formosanus* Shiraki in the United States is reported. Sixteen individual or isomeric mixtures of hydrocarbons were identified by gas chromatography–mass spectrometry. Hydrocarbon components consist of *n*-alkanes, 2-methylalkanes, 3-methylalkanes, internally branched monomethylalkanes on carbons 9–15, and dimethylalkanes. The predominant hydrocarbons have 27 carbons in the parent chain. Methyl-branched hydrocarbons are more abundant than *n*-alkanes. No qualitative differences were apparent in the hydrocarbon components of workers or soldiers from any of the four populations. Quantitative differences in the hydrocarbon components separate castes and populations into different concentration profiles. Stepwise discriminant analysis and canonical discriminant analysis were used to choose and display seven hydrocarbon components for workers and three for soldiers that best reveal the differences among populations. Within-population variation is low compared to the differences among populations. These results suggest that *C. formosanus* from Hallandale, Florida; New Orleans, Louisiana; and Lake Charles, Louisiana, are not related to those from Honolulu, Hawaii, and probably originated from other geographical locations.

Key Words—Cuticular lipids, chemotaxonomy, biogeography, founder principle, *Coptotermes formosanus*, Isoptera, Rhinotermitidae, insect quarantine, insect integument, canonical discriminant analysis.

[1] Isoptera: Rhinotermitidae.

INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, is considered to be one of the most voracious subterranean termites and a serious threat to wood in structures throughout its range. In addition to buildings, *C. formosanus* attacks live trees, creosoted transmission poles, structural pilings, and even underground utility cables. No accurate estimate of the economic impact of *C. formosanus* is available; it likely exceeds $100 million annually in the United States alone. Even though the common name implies that this species is from Formosa (Taiwan), *C. formosanus* is considered to be indigenous to mainland China (Kistner, 1985). It was introduced into Japan prior to 1600 and subsequently into Hawaii before 1907 (Swezey, 1914). Since its introduction into Hawaii, *C. formosanus* has been imported to Guam, Midway, Sri Lanka, Taiwan, South Africa, and the mainland United States (Su and Tamashiro, 1987).

Although many of the infestations in the continental United States were first noticed after 1965, *C. formosanus* colonies were most likely established following World War II. Active movement of military goods from the Pacific Theater to storage facilities in port cities of Houston and Galveston, Texas; New Orleans and Lake Charles, Louisiana; and Charleston, South Carolina, probably resulted in the establishment of *C. formosanus* in North America (Beal, 1987). There has been a significant "spread" of *C. formosanus* to other southern port cities including greater Fort Lauderdale, Florida; Memphis, Tennessee; Baton Rouge, Louisiana; and Mobile, Alabama. Because of the capacity of *C. formosanus* to infest wooden watercraft and wooden products transported on marine vessels, initial introductions have been near cities involved in shipping (La Fage, 1987).

Since 1980, indications of established infestations inland suggest this species may now be transported in infested wood products via domestic surface commerce (Chambers et al., 1988; La Fage, 1987; Sponsler et al., 1988). Despite the overall success of *C. formosanus* in infesting new areas, distribution is generally confined to regions where average temperatures for the coldest month do not fall below 4°C, and mean minimum daily temperatures for the same month are not less than 0°C. This includes locations ca. 36° north or south of the equator (Su and Tamashiro, 1987; Beal, 1987), and means potential for infestation of the highly populated, coastal metropolitan area of Los Angeles, California, north to the San Francisco Bay area.

With this potential threat to the most populous state in the United States, in addition to new locations along the entire Gulf Coast and much of the Atlantic Coast, effective quarantine procedures are imperative. Knowledge of how introductions of *C. formosanus* have been spread will help regulatory agencies formulate and/or reexamine quarantine policies and procedures. Has *C. formosanus*

been introduced numerous times from Asia or Hawaii, or once introduced, has it spread from port to port via domestic, maritime, or surface commerce? In this paper we report the identification of the hydrocarbon components in the cuticular wax of *C. formosanus* from four geographical locations. These baseline data are analyzed to determine whether similarities among cuticular hydrocarbon profiles can reveal the origin(s) of recent infestations of *C. formosanus*.

METHODS AND MATERIALS

Termites were collected from four geographically separated locations: Hallandale, Florida; New Orleans and Lake Charles, Louisiana; and Honolulu, Hawaii. Foraging parties from colonies were collected from the soil in Florida as described by Su and Scheffrahn (1986) or in Hawaii as described by Tamashiro et al. (1973). The seven different colonies from Florida all came from a single location (up to 5 km apart) in Hallandale. Six of the seven colonies from Honolulu were from the University of Hawaii campus; another came from Kaneoe on the island of Oahu. Hollow centers of live and dead trees were the source of *C. formosanus* from New Orleans and Lake Charles. Groups of workers (and soldiers from Lake Charles only) were removed from standing trees in these latter two sites as described by Waller and La Fage (1987). All collections were made within a 14-month period: New Orleans in August 1986, Lake Charles in November 1986, Hallandale in April 1987, and Honolulu in October 1987.

Termites were separated from wood debris and carton material, frozen, and subsequently dried in a desiccator. They were held dry at room temperature until hydrocarbons were extracted. Cuticular lipids were extracted by immersing 50–100 termites, as a group, in 10 ml of hexane for 10 min. After the termites were extracted, hydrocarbons were separated from other components and identified by gas chromatography–mass spectrometry (GC-MS) (Page et al., 1990). Routine quantification of cuticular hydrocarbon components was achieved with a Hewlett Packard 5890 gas chromatograph; all operating conditions were the same as described by Haverty et al. (1988), except that oven temperature was increased at 3°C/min to 320°C, with a final holding time of 11 min.

For each of the four *Coptotermes* populations, summary statistics (mean and standard deviation of the percent of each hydrocarbon component) were calculated separately for workers and soldiers. Similarity of hydrocarbon patterns or differences between the four populations was determined by stepwise discriminant analysis to select discriminating variables, followed by canonical discriminant analysis to provide two-dimensional displays of the chosen variables. The purpose of the discriminant analysis was to define a subset of quan-

titative variables (in this case percentage of each hydrocarbon component) that best revealed the differences among the populations. The program that we used (STEPDISC) employs stepwise selection of variables (SAS, 1985). Canonical discriminant analysis is a dimension-reduction technique related to principle component analysis and canonical correlation that derives a linear combination of quantitative variables (percentage of hydrocarbon components) that summarizes between-class variation. The program that we used (CANDISC) computes and tests Mahalanobis distances for pairwise comparisons of the populations (SAS, 1985). We assume that these distances are indicative of the degree of genetic similarity between populations.

RESULTS AND DISCUSSION

GC-MS analyses of the cuticular wax of representative samples of *C. formosanus* (one group of workers and/or soldiers from each location) indicate that all colonies and both castes from all four locations contain the same hydrocarbon components. All of the 16 major hydrocarbon components and/or isomeric mixtures (mean percent $\geq 0.1\%$ of the total hydrocarbon mixture) were characterized (Table 1, Figure 1). Hydrocarbon components consist of homologous series of *n*-alkanes, 2-methylalkanes, 3-methylalkanes, and single-component and isomeric mixtures of internally branched monomethylalkanes and dimethylalkanes. No unsaturated components were identified. With the exception of one dimethylalkane, hydrocarbon structures were previously identified in other insects by the authors (Haverty et al., 1988; Page et al., 1990).

The *n*-alkane composition is a continuous series from *n*-pentacosane to *n*-octacosane with *n*-heptacosane predominating. Both 3-methylalkanes and 2-methylalkanes were found. 3-Methylheptacosane (peak 11) occurs in significant quantities ($\geq 5\%$) whereas 3-methylpentacosane occurs in small amounts except in the colonies from Lake Charles, Louisiana. It is difficult to distinguish 2-methylalkanes from 4-methylalkanes (Blomquist et al., 1987); however, all are identified as 2-methylalkanes because there was no strong carbonium ion pair at M-71 : M-72, indicating that the 4-methylalkanes were not present (Table 1). 2-Methylalkanes are quite predominant in the hydrocarbon profiles of *C. formosanus*; they usually are much more abundant than the corresponding *n*-alkane of the same chain length. No single-component 5- or 7-monomethyalkanes are present in *C. formosanus*. However, significant quantities ($>0.5\%$ total hydrocarbon) of other internally branched monomethylalkanes occur in this species. The most abundant hydrocarbon component in the cuticular wax of *C. formosanus* is peak 9, a mixture of 9-, 11-, 13-methylheptacosane. Only two dimethylalkanes are produced by *C. formosanus*: 9,13-dimethylheptacosane and 13,15-dimethylnonacosane. The former coelutes with 2-methylheptacosane in

TABLE 1. PERCENT COMPOSITION OF HYDROCARBONS FROM WORKERS AND SOLDIERS OF *Coptotermes formosanus* SHIRAKI FROM FOUR DISJUNCT LOCATIONS IN THE UNITED STATES[a]

		Workers				Soldiers	
Peak[b]	Hydrocarbon[c]	Hallandale (N = 7)	New Orleans (N = 5)	Lake Charles (N = 9)	Honolulu (N = 7)	Lake Charles (N = 10)	Honolulu (N = 4)
1	*n*-C25	0.9 (0.3)	1.6 (0.3)	1.2 (0.4)	0.9 (0.1)	1.6 (0.5)	1.7 (0.9)
2	9-, 11-MeC25[d]	0.4 (0.4)	0.6 (0.3)	1.5 (0.2)	0.3 (0.4)	1.1 (0.5)	0.0 (0.0)
3	2-MeC25	8.6 (0.8)	7.8 (0.9)	14.7 (1.9)	8.3 (1.7)	8.5 (1.6)	4.0 (2.4)
4	3-MeC25	0.4 (0.3)	0.6 (0.1)	2.4 (0.5)	0.5 (0.3)	1.8 (0.5)	0.2 (0.3)
5	*n*-C26	1.0 (0.2)	2.1 (0.3)	1.2 (0.3)	1.2 (0.6)	2.1 (0.4)	3.1 (1.2)
6	9-, 11-, 13-MeC26[d]	1.3 (0.1)	1.3 (0.2)	1.5 (0.1)	0.8 (0.5)	1.2 (0.7)	0.1 (0.3)
7	2-MeC26	3.3 (0.4)	4.0 (0.4)	4.6 (0.6)	4.1 (0.6)	3.1 (0.4)	2.5 (0.8)
8	*n*-C27	4.9 (0.9)	5.9 (1.3)	4.1 (0.9)	8.9 (3.7)	11.0 (3.7)	27.7 (13.9)
9	9-, 11-, 13-MeC27[d]	31.7 (1.9)	29.8 (3.6)	28.5 (2.7)	27.1 (2.9)	25.4 (3.6)	17.9 (3.9)
10	2-MeC27 + 9,13-DimeC27	22.9 (1.4)	22.2 (2.1)	16.9 (1.6)	23.8 (2.0)	16.2 (2.6)	18.3 (6.2)
11	3-MeC27	6.5 (0.4)	6.8 (0.6)	7.8 (0.9)	7.2 (0.4)	8.9 (1.5)	6.4 (1.9)
12	*n*-C28	0.9 (0.5)	1.7 (0.2)	0.4 (0.2)	1.2 (0.6)	0.9 (0.5)	2.9 (0.9)
13	9-, 11-, 13-, 15-MeC28[d]	3.3 (0.3)	3.1 (0.3)	3.0 (0.4)	2.9 (0.2)	3.1 (0.8)	2.1 (1.2)
14	9-, 11-, 13-, 15-MeC29 + 13,15-DimeC31[d,e]	13.8 (0.7)	12.5 (0.9)	12.3 (2.0)	12.8 (2.3)	15.0 (5.5)	12.9 (1.9)

[a] Hydrocarbons are quantified as the mean percent (standard deviation) of the total hydrocarbon component.
[b] Peak numbers refer to peaks identified in Figure 1.
[c] This shorthand uses a descriptor for the location of the methyl group (X-Me) and the total number of carbons (CXX) in the hydrocarbon component, excluding methyl branch(es).
[d] A mixture of positional isomers; two or more components coelute in this peak.
[e] Because of incomplete separation of peaks 14 and 15 during routine quantification of components, both peaks are included as one value.

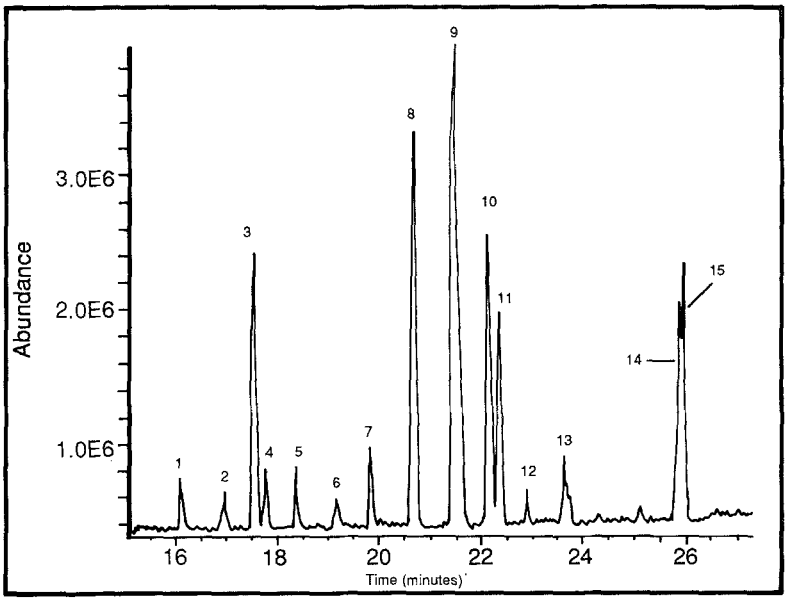

FIG. 1. Total ion chromatogram of the cuticular hydrocarbons from *Coptotermes formosanus* Shiraki collected from Honolulu, Hawaii. Numbers identify peaks listed in Tables 1 and 2.

peak 10 and occurs as a single isomer. The latter elutes as a shoulder of the 9-, 11-, 13-methylnoncosane peak (Figure 1).

The mass spectrum of peak 15 (Figure 2) is interpreted as arising from 13,15-dimethylnonacosane. Hydrocarbons with the methyl branches separated by a single methylene group are not common in insect hydrocarbons (Blomquist et al., 1987). The M-15 ion at *m/z* 421 indicates a hydrocarbon with 31 carbons. The larger fragments, which have a high odd/even ratio at *m/z* 267/266 and 239/238, are consistent with cleavage external to the two branching methyl groups (Figure 2). The fragments at *m/z* 224/225 and 196/197 have an even-to-odd ratio greater than one, consistent with cleavage internal to each methyl branch. The observed equivalent chain length (ECL) of this component (29.47) is slightly less than the ECL of this compound reported from *Solenopsis invicta* Buren (Nelson et al., 1980). However, dimethylalkanes with one methylene group separating the methyl substituents are known to produce diastereoisomers with different elution times yet nearly identical mass spectra (Pomonis et al., 1980). We examined a synthesized standard, 9,11-dimethylheptacosane, provided by J.G. Pomonis. On our system, two separate peaks eluted from this standard with ECLs of 27.4 and 27.7. The mass spectra of these two peaks

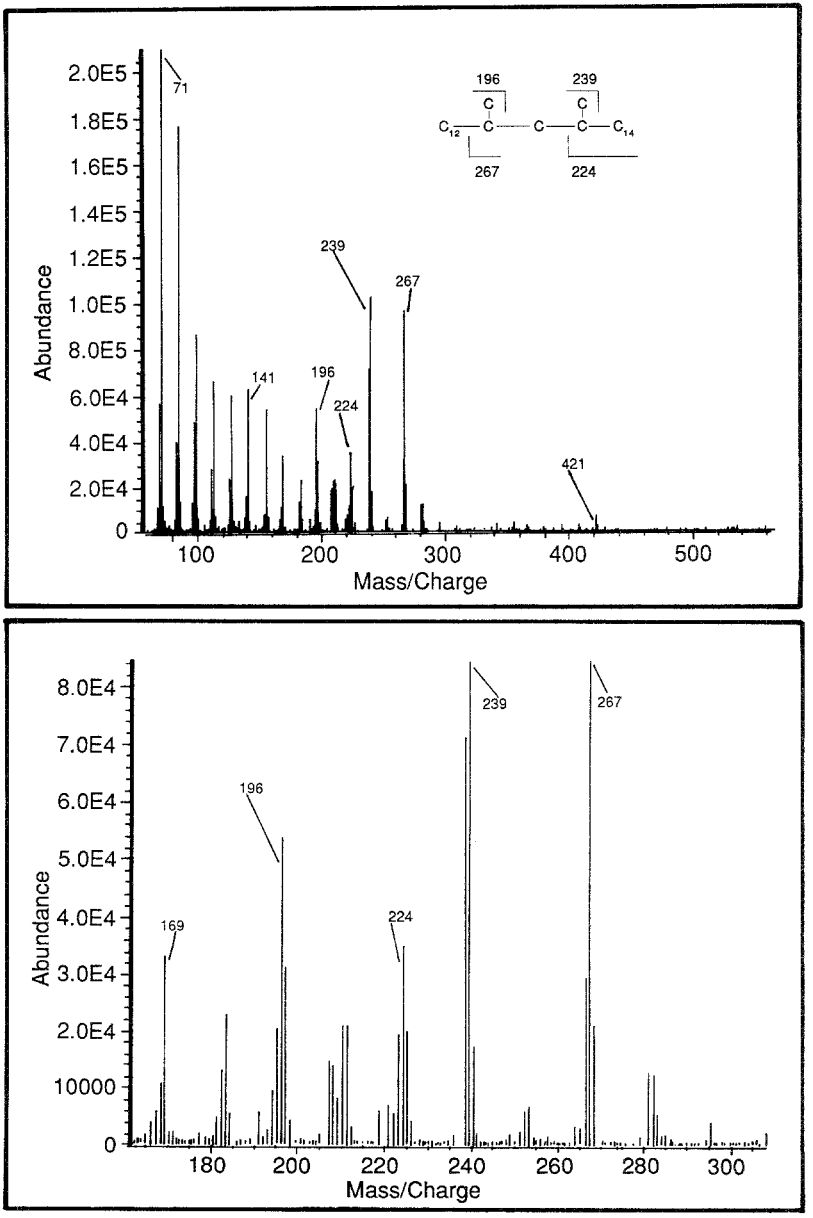

FIG. 2. EI mass spectrum of peak 15, Figure 1, identified as 13,15-dimethylnonacosane.

were identical. Insects apparently make one or the other diastereomer: *C. formosanus* makes the early-eluting compound (confirmation provided by D.R. Nelson and J.G. Pomonis).

The cuticular hydrocarbons of *C. formosanus*, as is the case for many insect species, consist of complex mixtures of straight-chain and methyl-branched saturated components (Blomquist et al., 1987). The relatively large number of potential components, ease of analysis, and species-specific compositions make them attractive characters for use in chemotaxonomy (Carlson and Bolten, 1984; Carlson and Brenner, 1988; Gastner and Nation, 1986; Haverty et al., 1988; Howard et al., 1988; Lockey, 1982; Page et al., 1990; Vander Meer, 1986).

Description of the cuticular hydrocarbons of other species of *Coptotermes* would be a logical extension of this work, but our goal in this study was not to test the taxonomic status of the introductions of *C. formosanus* into the continental United States. Rather, we examine the potential for using this set of quantitative chemical characters to determine the similarity between the hydrocarbon profiles within and among the sampled geographic locations. Our underlying assumption is that colonies of *C. formosanus* with quantitatively similar cuticular hydrocarbon profiles are likely to be more closely related, i.e., originating from the same geographical source, than those that are less similar.

Discriminant analysis of the proportions of the cuticular hydrocarbons of workers identified seven hydrocarbon components that reasonably separate the four populations: peaks 2–8 (Table 1, Figure 1). For the two locations for which we had soldiers, only three hydrocarbon components were necessary: peaks 3, 4, and 14. With these hydrocarbon variables, we were able to display the differences with plots of hydrocarbon proportions along two axes of canonical discriminant space (Figure 3) and to test the similarity between locations. Statistical analysis of Mahalanobis distances for all possible comparisons of workers from these four geographic locations indicates that all distances are statistically different from zero (Table 2). The same is true of the distance of hydrocarbon profiles of soldiers from Lake Charles and Hallandale; the estimated Mahalanobis distance is 6.0 and is statistically different from zero at the 0.0001 level. In other words, colonies of *C. formosanus* from each of the four geographic locations can be separated from colonies from all of the other sites on the basis of concentrations of cuticular hydrocarbon components. Only slight overlap occurred between the Hallandale and Honolulu populations using just the first two canonical variates.

Carlson and Brenner (1988) used GC-MS followed by principal component analysis to identify and analyze the cuticular hydrocarbons of *Blattella germanica* (L.) from several distant geographical locations and from museum specimens. Their primary objective was to develop a technique for identifying immatures of the closely related species, *B. germanica* and *B. asahinai* Mizukubo, for which no diagnostic morphological characters exist. For *B. german-*

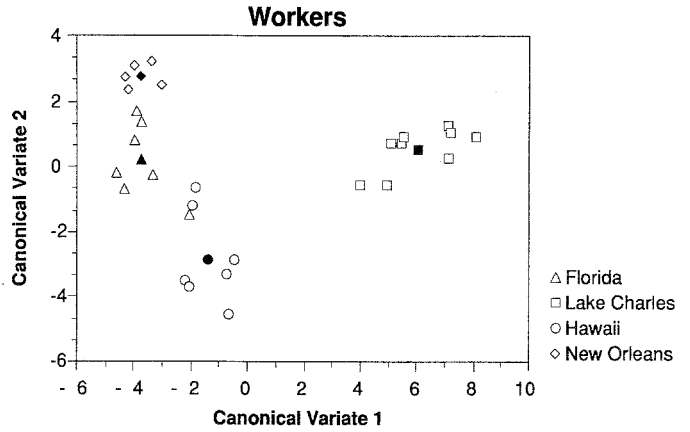

FIG. 3. Plot of colonies of *C. formosanus* from four different locations along two axes of canonical discriminant space. Open characters represent actual data points; closed characters represent the mean value for the population.

ica, they found "no appreciable difference . . . in patterns of individuals from the populations examined. . . ." The chromatographic patterns of field-collected specimens from Washington, D.C., Alaska, Florida, and other Gulf Coast states were similar to those of New Caledonia from museum collections. Thus for *B. germanica*, analysis of concentrations of cuticular hydrocarbons does not appear to be useful for determining the origin of populations, at least from the sample reported by Carlson and Brenner (1988).

This is the first use of cuticular hydrocarbons to ascertain similarities or differences between populations (in our case collections of separate colonies) of the same species from disjunct geographic locations. From our previous

TABLE 2. MAHALANOBIS DISTANCE BETWEEN POPULATIONS OF WORKERS OF *Coptotermes formosanus* SHIRAKI FROM FOUR DISJUNCT LOCATIONS IN THE UNITED STATES[a]

Location	Honolulu	Lake Charles	New Orleans
Hallandale	4.64	9.92	4.19
Honolulu		8.29	6.11
Lake Charles			10.22

[a] Probabilities of Mahalanobis distance test the difference between each value and zero. All distances in this table are statistically greater than zero at the 0.005 level.

experience (Haverty et al., 1988), we know that within a species or hydrocarbon phenotype of the dampwood termites, *Zootermopsis* (Hagen), there are statistically significant differences between colonies or castes for many of the hydrocarbon components in each species' profile. No single component, however, can be used to separate all four of the geographic locations of *C. formosanus*. Stepwise discriminant analysis allows us to use a selection of the hydrocarbon components to separate these geographic locations.

We believe that the utility of cuticular hydrocarbons for determining similarity of insect populations has promise. Clearly, the population from Lake Charles is very different from those from the other locations. This would support the assertion that the introduction of *C. formosanus* into Louisiana was from two separate sources (La Fage, 1987). It also appears that the Honolulu population, at least the insects we sampled from the Manoa Valley in Honolulu, was probably not the original source of infestations in either New Orleans, Lake Charles, or Hallandale. These "invasions" likely originated from elsewhere in the Pacific Theater. The affinity of the infestation from Hallandale, Florida, is least clear. The hydrocarbon profile is significantly different from both Honolulu and New Orleans. Given that the site of this infestation is in an area of eastern Florida frequented by large, ocean-going pleasure craft, either origin is likely. With our present data base, cuticular hydrocarbon profiles have not allowed us to determine whether the more recent infestation in Hallandale, Florida, resulted from international or domestic maritime or surface commerce.

This study points to the need for additional information and sampling. We need to analyze the variation in hydrocarbon components of *C. formosanus* over a greater geographical range. The samples used in this study are from relatively small areas within their respective geographic locations. This cannot be helped in Hallandale or Lake Charles because of the restricted nature of the local distribution. Similarity within a geographic area may, in fact, simply represent a founder event. A much more dispersed sample could be taken in Hawaii, all over the island of Oahu and other locations on the neighboring islands, where *C. formosanus* is now established (Tamashiro et al., 1987). Such a biogeographical study would greatly assist the evaluation of hydrocarbon profiles as an indicator of the origin of *C. formosanus* populations.

We cannot yet rule out that the differences in cuticular hydrocarbon profiles among the four populations of *C. formosanus* may be due to diet (Prestwich, 1983). Hydrocarbons are almost exclusively manufactured by insects (Blomquist et al., 1987); however, we do not know how food source affects biosynthesis of the various components in cuticular hydrocarbons or if differences in concentration may result. The insects from Lake Charles were collected from the interior of bald cypress, *Taxodium distichum* Rich., whereas those from New Orleans came from a variety of living trees (see La Fage, 1987). The colonies from Hallandale and Honolulu were collected from traps in the soil

and could have been feeding on numerous structural materials as well as other vegetative sources.

An increasing body of knowledge is accumulating that indicates hydrocarbon profiles are species-specific (Carlson and Brenner, 1988; Howard et al., 1982, 1988; Haverty et al., 1988; Page et al., 1990; Vander Meer, 1986). Qualitative differences in cuticular hydrocarbons have led us and our colleagues to identify a new subspecies, or possibly species, of *Zootermopsis* (Haverty and Thorne, 1989) and to find a morphological character for unequivocal identification of the three extant species of *Zootermopsis* (Thorne and Haverty, 1989). Cuticular hydrocarbons will help us corroborate or contradict current taxonomy based on the morphology and feeding behavior of the refractory genus of cone beetles, *Conophthorus* Hopkins (Page et al., 1990). In this paper we have introduced yet another biologically meaningful use of cuticular hydrocarbon data. We anticipate that the combination of GC-MS with discriminant or cluster analysis followed by canonical discriminant analysis will prove more powerful and precise than GC-MS analyses alone in resolving statistical comparisons of proportions or ratios of pairs of individual hydrocarbon components (Carlson and Bolten, 1984; Carlson and Service, 1980; Howard et al., 1988). Such clustering techniques will assist in separating closely related populations within a species or closely related and morphologically indistinguishable species when no qualitative differences in hydrocarbon profiles are apparent (Carlson and Brenner, 1988; Howard et al., 1988).

Thus far, isozyme analysis has provided limited information on the relatedness of *C. formosanus* populations in Florida, Louisiana, and Hawaii (A.K. Korman, personal communication). Additional potential methods of examining similarity among populations would be to decipher the genetic code (DNA hybridization) or to characterize end products of the genetic code, such as morphology, cuticular hydrocarbons, and agonistic behavior (Su and Haverty, in preparation). From one or more of these methods we should be able to establish relationships among populations of *C. formosanus* throughout its distribution. Future studies will involve further characterization of the cuticular hydrocarbons of additional collections of *C. formosanus*, and additional species within this genus, to evaluate quantitatively intra- and interspecific variation in hydrocarbon patterns. It is hoped, further study will clarify and/or validate the use of cuticular hydrocarbons for separating species or determining the origin of introduced populations of the Formosan subterranean termite.

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DOES THE IMPORTED CABBAGEWORM, *Pieris rapae*, USE AN OVIPOSITION DETERRING PHEROMONE?

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Abstract—Eggs of *Pieris rapae* (L.) contain a water- and methanol-soluble oviposition-deterring pheromone (ODP) that is avoided by ovipositing females offered treated and untreated cabbage leaves in a choice situation. Water extracts of female accessory glands also deter oviposition. Egg washes and gland extracts stimulate a contact chemoreceptor in sensilla on the fore tarsi. Electroantennogram (EAG) recordings show that antennal receptors respond to volatiles from conspecific eggs, but not to volatiles from eggs of *Mamestra brassicae*. No differences were detected between North American and European insects with regard to production and perception of ODP. These results differ from conclusions in the literature based upon field observations. It is concluded that this discrepancy in due to methodological differences.

Key Words—*Pieris rapae*, Lepidoptera, Pieridae, imported cabbageworm, oviposition deterring pheromone, egg dispersion, chemoreception.

INTRODUCTION

Previous studies provide conflicting evidence about whether the imported cabbageworm, *Pieris rapae* (L.)(Lepidoptera; Pieridae) marks its eggs with an oviposition-deterrent pheromone (ODP) and thereby promotes an even distribution of eggs among available host plants. Rothschild and Schoonhoven (1977) concluded from preliminary experiments under laboratory conditions that *P. rapae* discriminates between a cabbage leaf from which conspecific eggs had been removed and a "clean" control leaf. Klijnstra (1985a, p. 230), on the basis of 17 laboratory experiments, concluded somewhat equivocally that "females may tend to lay fewer eggs on egg laden plants." However, several other studies

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based on field (Kobayashi, 1965; Ives, 1977; Root and Kareiva, 1984) or laboratory (Traynier, 1979) observations failed to show discrimination against egg-containing plants. In some cases even a certain degree of clumping has been reported (Harcourt, 1961; Jones, 1977).

These conflicting conclusions may be caused by any of the following reasons. (1) The preliminary results used to infer the presence of an ODP in *P. rapae* (Rothschild and Schoonhoven, 1977; Klijnstra, 1985a) may misrepresent reality. (2) Geographical differences may exist between the insects used in different studies. (3) Methodological differences may have caused different results. To identify the cause of the discrepancy, we have conducted a more detailed study of whether *P. rapae* employs an ODP and thus deters conspecific females from ovipositing in the neighborhood of previously laid eggs. Additionally, oviposition responses between European and American insects were compared to detect possible geographical differences in the use of an ODP.

METHODS AND MATERIALS

Insects. A colony of *P. rapae*, established from insects caught in the wild in the Netherlands, was kept for about 25 generations in a greenhouse (conditions: 19–25°C, photoperiod light–dark = 16–8, relative humidity: 65–80%) on cabbage (*Brassica oleracea gemmifera* cv. titurel). Insects of American origin were kindly provided by Dr. J.A.A. Renwick from a culture derived from females collected near Ithaca, New York, and kept for about 20 generations in a greenhouse on cabbage (*B. oleraccea gemmifera* cv. Golden Acre). All butterflies were fed on artificial flowers containing 10% sugar solution.

Egg Washes. Eggs laid by European or American insects were carefully brushed off the leaf surface and shaken for 5 min with methanol or water (500 eggs/1 ml solvent). Egg washes were stored at 3°C for one to eight days before use.

Accessory Gland Extracts. Water extracts of female accessory glands were obtained by bathing two pairs of excised glands in 1 ml water for 2 hr. Washes from comparable amounts of female fat body were used as a control. For further details see Behan and Schoonhoven (1978).

Behavioral Tests. Two cabbage leaves (10–15 cm diam.) picked from the same plant were offered in an 80 × 100 × 100-cm cage to 4–10 butterflies, 2–12 days old. One leaf was sprayed with 1 ml egg wash or accessory gland extract, and the other was treated with solvent only (control). To avoid position effects, leaves were transported periodically. In addition to natural light each cage was illuminated from above by a mercury vapor lamp. Each test lasted from 10:00 AM until 2:00 PM. ODP indexes were calculated using the formula: % deterrence = $[(A - B) \times 100]/(A + B)$, where A and B are the numbers

of eggs laid on control and treated leaves, respectively. An ODP index of 100% indicates that butterflies laid all eggs on the control leaf, whereas 0% indicates no preference.

Electrophysiology. Forelegs of 1- to 4-day-old female were cut at the middle of the tibia and a silver wire (ϕ 30 μm) was pushed into the distal part of the tibia and connected to the input of a high input impedance preamplifier (Hodgson et al., 1955; Drongelen, 1979). The stimulus capillary contained 3 mM NaCl (control) supplemented by either egg water wash (conc.: 500 eggs/ml) or 10 mM singrin, or 30 mM sucrose. The indifferent electrode was in contact with the stimulus solution. Tarsal B-hairs were stimulated by positioning the stimulus capillary over the tip of a sensillum for 1.5–2 sec. For further details see Ma and Schoonhoven (1973).

Electroantennograms (EAGs) were registered from excised antennae positioned between two micropipets and exposed to a constant airstream (1800 ml air/min, 20°C, humidified). The stimulus consisted of a 1 ml/sec airstream lasting 2 sec, which has passed along a piece of filter paper containing either 25 μl 0.1% *cis*-3-hexen-1-ol in paraffin oil (standard), or 0.2 g *P. rapae* eggs, or 0.4 g *Mamestra brassicae* eggs. Responses to eggs were expressed as percentages of responses to standard stimuli.

RESULTS

Behavioral Experiments. When butterflies were offered cabbage leaves treated with egg wash, together with "clean" control leaves, they showed a strong oviposition preference for control leaves (Table 1). Thus, ovipositing females apparently are able to detect the presence of a chemical extracted from

TABLE 1. CHOICE BEHAVIOR IN OVIPOSITING BUTTERFLIES WHEN EXPOSED TO ODP EXTRACTS

Stimulus	Replicates (*N*)	Total eggs (*N*)		ODP deterrence (%)
		Treated leaves	Control leaves	
U.S.A. insects				
Egg wash U.S.A.	12	185	956	68[a]
Wageningen insects				
Egg wash Wageningen	9	249	1453	71[a]
Accessory gland extract	10	870	1302	20[a]
Fat body extract	4	318	268	−8 (N.S.)

[a] Different from 0, $P < 0.01$ (chi-square test).

the eggs. Insects originating from the United States showed the same responses as insects collected in Europe. A water extract from female accessory glands was weakly, although significantly, deterrent, whereas a fat body extract, which served as the control, did not significantly affect female behavior.

Electrophysiological Experiments. Sensitivity to water solutions containing ODP has been observed in the tarsal sensilla of the large white butterfly, *Pieris brassicae,* (Behan and Schoonhoven, 1978), the apple maggot fly (Crnjar and Prokopy, 1982), and the European cherry fruit fly (Hurter et al., 1987). In this study, the tarsal sensilla of *P. rapae* (Wageningen culture) displayed an intense electrophysiological response to egg washes; this response arose predominantly in one chemoreceptive neuron (Table 2). The same cell also exhibited a strong, albeit slightly lower, response to an accessory gland extract. This lower response may indicate that the gland extract (prepared from two paired glands per milliliter of solvent) contained less ODP than the egg wash (obtained from 500 eggs/ml).

Behavioral observations on *P. brassicae* females suggested that the ODP was perceived from some distance (Rothschild and Schoonhoven, 1977; Klijnstra, 1985b), indicating the presence of airborne cues. In a previous study, EAG recordings revealed that the antennae of *P. brassicae* females are stimulated by egg volatiles (Behan and Schoonhaven, 1978). To determine whether the antennae of *P. rapae* show a similar response, EAGs were recorded using intact egg odors and pieces of filter paper with egg wash. Eggs of *P. rapae* clearly evoked antennal responses, which were for European insects on average 10.3% ($\pm$ 0.9; $N = 21$) of the response elicited by the (highly stimulatory) standard stimulus (hexenol, see Methods and Materials), and 11.4% ($\pm$ 1.0; $N = 22$) for American insects. In contrast, neither eggs of *M. brassicae* (0.1% $\pm$ 0.1; $N = 8$) nor washes of *P. rapae* eggs produced a reaction in *P. rapae* antennae. Thus no differences in antennal responses occur between American and European insects.

TABLE 2. MEAN NUMBER OF IMPULSES ($\pm$SEM) IN FEMALE TARSAL SENSILLA DURING FIRST SECOND OF STIMULATION WITH VARIOUS EXTRACTS[a]

Stimulus	Impulse frequency $\pm$SE
Egg wash	83.5 $\pm$ 9.3a
Accessory gland extract	53.9 $\pm$ 5.6b
Fat body extract	14.9 $\pm$ 2.6c
10 mM NaCl	15.1 $\pm$ 2.6c

[a]Legs and stimuli were derived from the Wageningen colony. Means with different letters are significantly different ($P \leq 0.01$), $N = 30$.

DISCUSSION AND CONCLUSIONS

The behavioral evidence presented unequivocally demonstrates that *P. rapae* butterflies avoid laying eggs on plants that have been treated with ODP containing egg washes. This phenomenon has been described before for *P. brassicae* (Rothschild and Schoonhoven, 1977; Klijnstra, 1985b), which lays its eggs in batches of up to 100 eggs. *P. rapae,* however, oviposits single eggs. Despite a lower risk of conspecific food competition, it appears that *P. rapae* also has developed a chemical signal that aids the ovipositing female to avoid laying eggs on occupied plants.

The behavioral evidence for the presence of an ODP in *P. rapae* is corroborated by the finding that egg washes of this species stimulate a chemoreceptor in the female's tarsal sensilla. The same cell responds, although at a lower intensity, to extracts from the female's accessory glands. These extracts also inhibit oviposition to a certain degree, and therefore the glands likely contain the production sites and/or storage places of ODP.

Antennal responses measured by EAGs indicated that *P. rapae* possesses olfactory cells that can be stimulated by volatiles associated with conspecific eggs, but that are insensitive to odors of eggs from *M. brassicae,* a noctuid also occurring on cabbage plants. Presumably the ability to perceive airborne chemicals liberated from conspecific eggs plays an additional role in increasing the dispersion of *P. rapae* eggs.

Given that *P. rapae* employs oviposition-deterring chemicals, why have some previous studies failed to detect an ODP? Since the reports cited were all based on observations of *P. rapae* in North America and Australia, where it was introduced more than 100 years ago (Scudder, 1887; Elton, 1958) and where *P. brassicae* does not occur, these populations might have lost their ability to perceive conspecific eggs. It could be argued that, although during evolution ODPs were primarily developed to reduce oviposition by an insect near its own previously laid eggs (Roitberg and Prokopy, 1987) the deposition and perception of ODP by *P. rapae* is maintained mainly because it reduces competition with *P. brassicae.* The fact that ovipositing *P. brassicae* females avoid washes of *P. rapae* eggs, and vice versa (Schoonhoven et al., 1989), is in agreement with the supposition of a heterospecific function of the ODP. However, the similarity in response of the Dutch and American populations to egg washes argues strongly against this explanation.

It must be concluded, therefore, that the incongruity between some literature reports and the results from the present study are due to methodological differences. The conclusions reached by Harcourt (1961), Jones (1977), Ives (1978), and Root and Kareiva (1984) were all based on observations under natural conditions. Although ODPs in some other insects have been found to act under field conditions, for instance by increasing dispersal activity (Roitberg

et al., 1984; Klijnstra and Schoonhoven, 1987), it may be difficult in the complexity of a natural situation to detect such activity statistically, especially when egg densities are relatively low. The observation by Kobayashi (1965), that at low densities *P. rapae crucivora* egg distributions show some degree of aggregation, whereas with the increase of density eggs are laid more evenly and extensively, agrees with the idea that egg density affects distribution pattern. Other environmental factors, such as host distribution patterns (Singer, 1986) and variations in host quality (Averill and Prokopy, 1989), also may override ODP effects. Kellogg (1985) found that *Pieris sisymbrii* possesses egg-avoiding behavior, which, however, may be easily masked by variation in host encounters. Additionally, unequivocal evidence for egg avoidance has been observed to operate in nature in two other pierid species that lay their eggs singly (Wiklund and Åhrberg, 1978; Shapiro, 1980). The present study, utilizing laboratory observations under highly standardized conditions and using large amounts of ODP, may have been able to detect behavioral responses to ODP that are relatively invisible in a complex and variable natural situation.

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OVIPOSITION STIMULANTS IN THE COCCOID CUTICULAR WAXES OF *Aphytis yanonensis* DE BACH & ROSEN

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Abstract—*Aphytis yanonensis* De Bach & Rosen, a parasitic wasp of the arrowhead scale, *Unaspis yanonensis* (Kuwana), was introduced to Japan to control *U. yanonensis*. *A. yanonensis* recognizes a host insect by antennal contact and deposits eggs on the insect body underneath the scale. Ovipositional behavior is induced by perceiving the cuticular wax of the host insect, *U. yanonensis*, and other coccoids. Chemical composition of the cuticular wax was analyzed and oviposition stimulants were isolated following a bioassay using *A. yanonensis*.

Key Words—*Aphytis yanonensis*, Hymenoptera, Aphelinidae, oviposition stimulant, kairomone, *Unaspis yanonensis*, Homoptera, Diaspididae, cuticular wax.

INTRODUCTION

Two spcies of parasitic wasps of the arrowhead scale, *Unaspis yanonensis* (Kuwana), were introduced to Japan by a mission sent to China in 1980 by the Shizuoka Prefectural Government and the Ministry of Agriculture, Forestry and Fisheries (Nishino and Takagi, 1981). Identified as *Aphytis yanonensis* De Bach and Rosen and *Cocobius fulvus* Compere and Annecke in 1982 (De Bach and Rosen, 1983), both parasitic wasps are considered potentially highly effective natural enemies against the arrowhead scale in Japan and were first released in Shizuoka Prefecture to control *U. yanonensis* in 1981 (Furuhashi and Nishino, 1983).

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A. yanonensis is ectoparasitic and deposits eggs under the scales of several developmental stages of *U. yanonensis*, i.e., second instar, immature adult, and mature female. Size and shape of the host scale do not appear to influence the wasp's ovipositional behavior. The average total fecundity per *A. yanonensis* female at 25°C was 17.3 progeny, but the number increased with a larger host such as *Aspidiotus nerii* Bouche (= *hederae*) (Furuhashi and Nishino, 1983). Yukinari (1987) showed that *A. yanonensis* laid eggs on *Unaspis euonymi* (Comstock) and *Lepidosaphes cupressi* (Coleman) in the laboratory when it was confined with the coccoids on their host plants. These findings, and the use of *Hemiberlesia lataniae* (Signoret) on pumpkin as a substitute host species for the rearing of *A. yanonensis* at the Kuchinotsu Branch of the Fruit Tree Research Station in Japan, prompted us to investigate the host range of *A. yanonensis*. The correlation of the ovipositional stimulant, a kairomone, present in the cuticular wax of the coccoids and the possible range of the host insects for *A. yanonensis* are of great interest.

METHODS AND MATERIALS

Insects. The latania scale, *H. lataniae*, was successively reared on pumpkin. A pumpkin with mature latania scale was placed on top of a fresh pumpkin onto which its hatched first instars (crawlers) dropped and colonized.

Aphytis yanonensis was introduced to a container with mature latania scale raised on pumpkin, and the F_1 generation emerged about 15 days later. Adult females were fed honey for one day after emergence and then were tested for oviposition behavior.

Observation of Oviposition Behavior of A. yanonensis *on* U. yanonensis *and Other Coccoids.* A hole 30 mm in diameter cut in the center of a 5-mm-thick glass plate was covered on both sides with glass. An intact coccoid was affixed inside the cover glass with honey. A female *A. yanonensis* was introduced into the space, 30 mm in diameter and 5 mm in height, and her behavior toward the coccoid was observed with a binocular microscope. The ovipositional response of *A. yanonensis* to other coccoids also was bioassayed in the above manner. A sample from the coccoid cuticular wax, synthetic waxes, and triglycerols were melted onto a glass plate and placed inside the space with *A. yanonensis* for bioassay in the same way as above.

Extraction and Purification of Cuticular Wax. Live coccoids were detached from host plants and immersed in chloroform. The chloroform solution was filtered and concentrated in vacuo to give a white powdery wax. The crude wax was chromatographed on silicic acid and eluted with benzene. The purified wax showed a single spot at R_f 0.5 (silica gel TLC developed with benzene).

Methanolysis of Cuticular Wax. Methanolysis of the wax was carried out

in 5% hydrogen chloride in methanol at room temperature. The products were purified on a silicic acid column and the fatty acid methyl esters and alcohols were analyzed by gas–liquid chromatography.

Extraction and Purification of Cuticular Wax from Hemiberlesia lataniae. Chloroform extract of the latania scale (2 g) gave 390 mg of waxy material. This was chromatographed on silisic acid (Wakogel C-200 20 g) and successively eluted with hexane, a 1 : 1 mixture of hexane and benzene, and 25% hexane in benzene and benzene alone. The eluate with the 1 : 1 mixture of hexane and benzene and the benzene eluate were purified separately on HPLC (nucleosil) by elution with 10% ether in hexane. The wax fraction with high oviposition stimulant activity (207 mg, 52.5% R_f 0.45 on silica gel TLC developed with benzene) was further separated into 16 fractions on HPLC (ODS; 2 × 200 mm) by eluting with 20% chloroform in acetonitrile. Two compounds (48 mg, 17.5% R_f 0.66 and R_f 0.72) obtained from the eluate with the 1 : 1 mixture of hexane and benzene were also analyzed.

Instrumental Methods. The gas–liquid chromatogram (GLC) was obtained using a Shimadzu 14AFP with a CBM 65 capillary column (0.23 mm × 25 m, 65% methylphenyl silicon). Mass spectra (EI at 70 eV) connected with a gas–liquid chromatography (OV-17, 1 m at 200–300°C, 10°C/min) inlet were obtained by a JEOL JMS-DX 303. FD mass spectra were obtained by a JEOL JMS-SX 102.

RESULTS

Oviposition Behavior of A. yanonensis. Typical initial behavior for the female was random walking and intermittent short flights. Antennal flagellation began when the female encountered a coccoid and moved onto it. The female then moved with antennal flagellation from the central part of the scale to its margin. When walking around the scale's margin, the female alternately moved backward and forward at different angles, repeating this several times and making a radial pattern. Thereafter, she began ovipositor drilling from the edge of the insect, moving backward at the same time.

Similar behavior was induced in the *A. yanonensis* female by a piece of scale cut from intact scale insects or cuticular wax. Such behavior was not induced, however, toward scale debris after immersion in chloroform. Table 1 shows the response of *A. yanonensis* to various coccoids. Three *Ceroplastes* species were not stimulative to *A. yanonensis*. Rates of antennal flagellation and oviposition drilling were calculated from the number of insects who performed these actions to the total number whose behavior was observed (Table 2).

Analysis of Cuticular Wax from Coccoid Insects. The three nonstimulating

TABLE 1. OVIPOSITIONAL BEHAVIOR OF *A. yanonensis* TO INTACT COCCOIDEA

Intact Coccoidea species	Ovipositional behavior observed		
	Flagellation	Drilling	Egg deposited
Diaspididae			
Unaspis yanonensis ♀	+	+	+
Unaspis yanonensis ♂	+	+	+
Unaspis euonymi	+	+	+
Pseudaulacaspis prunicola	+	+	?
Lepidosaphes cupressi	+	+	+
Lopholeucaspis japonica	+	+	?
Hemiberlesia lataniae	+	+	+
Pseudaonidia duplex	+	+	+
Comstockaspis perniciosa	+	+	?
Coccidae			
Ericerus pela ♂	+	+	−
Ceroplastes rubens	(+)[a]	−	−
Ceroplastes ceriferus	(+)	−	−
Ceroplastes japonica	(+)	−	−
Margarodidae			
Icerya purchasi ♀	+	+	−

[a] A few showed brief antennal contact.

TABLE 2. DIFFERENCE IN ANTENNAL FLAGELLATION AND OVIPOSITOR DRILLING RATES BY *A. yanonensis* TO INTACT COCCOIDEA

Coccoidea	Flagellation rate (%)[a]	Drilling rate (%)[b]
Diaspididae		
Unaspis yanonensis	69.6	84.4
Unaspis euonymi	65.9	74.1
Hemiberlesia lataniae	59.3	68.8
Lepidasaphes cupressi	63.1	63.4
Pseudalonidia duplex	75.0	60.0
Lopholeucaspis japonica	64.3	66.7
Pseudaulacaspis prunicola	61.3	57.9
Coccidae		
Ceroplastes rubens	0.025	0
Ceroplastes japonicus	0.014	0

[a] (No. of wasps showing flagellation/No. of wasps showing antennal contact) × 100.
[b] (No. of wasps showing drilling/No. of wasps showing flagellation) × 100; average of 10 *A. yanonensis* responses.

Ceroplastes species deposit a thick cuticular wax known to consist of a complex mixture of terpenoids, wax esters, alcohols, and fatty acids (Miyamoto et al., 1979; Pawlak et al., 1983; Tempesta et al., 1983). All other coccoid cuticular wax actively stimulated the ovipositional behavior of *A. yanonensis* during bioassay (Table 3). These waxes were found to be a mixture of wax esters with a composition similar to the cuticular wax of *U. yanonensis* (Hashimoto et al., 1971). The major component of the cuticular wax was analyzed by hydrolysis and gas chromatography (Table 4). From *Ericerus pela* (Westwood) the major components were wax esters including hexacosyl hexacosanoate (55.2%), hexacosyl tetracosanoate (22.3%), and hexacosyl octacosanoate (16.6%) (Takahashi and Nomura, 1982). The wax ester mixture was high in stimulant activity, but synthetic hexacosyl hexacosanoate demonstrated no stimulant activity to ovipositor drilling behavior.

The latania scale was found to be a good substitute host for rearing *A. yanonensis* in the laboratory. The major component (52.5%) of the latania scale wax was a mixture of triglycerols of moleclar weight from 666 to 890 (Figure 1): these consist mostly of mixed triglycerols with a fatty acid composition of lauric (11.83%), myristoleic (6.00%), myristic (44.13%), palmitoleic (10.37%), palmitic (16.45%), oleic (9.06%), and stearic (2.10%) acids. From the mass spectra (EI and FD) of the 16 fractions on an ODS column and the composition of fatty acids in the triglycerols, most of the structure was assigned as shown in Table 5. The synthetic triglycerols used for the identification of the

TABLE 3. OVIPOSITIONAL STIMULATION OF CUTICULAR WAX TO *A. yanonensis*[a]

Cuticular wax from	Behavioral response	
	Antennal flagellation (%)	Ovipositor drilling (%)
Unaspis yanonensis	100	100
Unaspis euonymi	100	100
Pseudaulacaspis prunicola	100	100
Hemiberlesia lataniae	100	50
Lepidasaphes cupressi	50	10
Ericerus pela	100	100
Hexacosyl hexacosanoate[b]	80	0
Triolein[b]	60	30
1-Palmito-dimyristin[b]	60	50
2-Oleo-dimyristin[b]	50	40
TG mixture[c]	90	80

[a] Rate of flagellation and drilling was calculated as in Table 2. Ten females for each sample were used.
[b] Synthetic compound.
[c] A mixture of equivalent amount of synthetic triglycerols; triolein, 1-palmitodimyristin, 2-oleo-dimyristin, tricaprylin, tricaprin, trilaurin, trimyristin, and tripalmitin.

TABLE 4. MAJOR COMPONENT OF COCCOIDEA CUTICULAR WAX

Species	Wax ester (carbon number of acid − carbon number of alcohol)
Diaspididae	
Unaspis yanonensis ♀	26-32 (5%), 28-32 (12%), 30-32 (47%), 32-32 (35%)
Unaspis yanonensis ♂	30-32 (42%), 32-32 (46%), 34-32 (12%)
Unaspis euonymi ♂ ♀	30-32 (25%), 32-32 (50%), 34-32 (25%)
Pseudaulacaspis prunicola ♂ ♀	30-32, 32-32, 34-32, 34-34 (major components); 28-28, 28-30, 28-32 (minor components)
Lepidasaphes cupressi ♂ ♀	30-32 (40%), 32-32 (44%), 32-34 (0.8%)
Hemiberlesia lataniae ♂ ♀	Mixed triglycerols (major components)
Coccidae	
Ericerus pela ♀	24-24 (3.1%), 24-26 (22.32%), 26-26 (55.2%), 28-26 (16.6%), 28-28 (3%)
Margaroidae	
Icerya purchasi ♀[a]	26-24 (13.8%), 26-26 (43.6%), 28-26 (25.2%), 28-28 (11.5%), 30-28 (6.0%)

[a] Extract from egg sac.

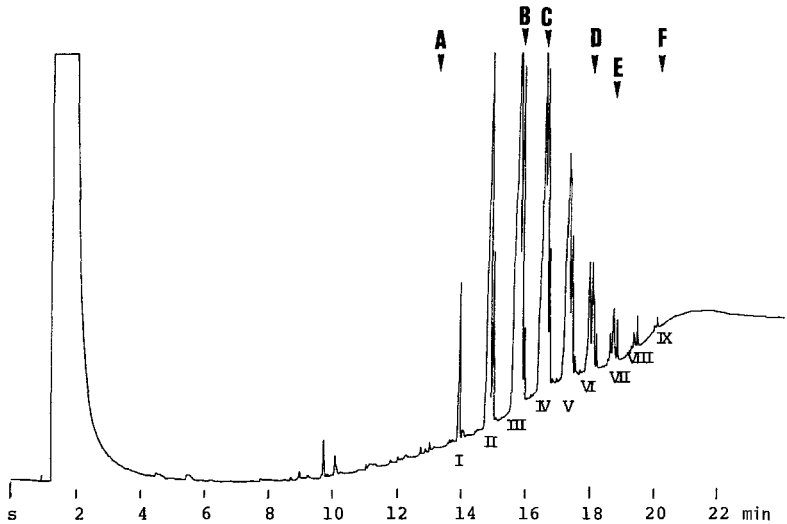

FIG. 1. Gas chromatogram of the triglycerols from *H. lataniae* cuticular wax. A Shimadzu 14APF with 65% methyl phenylsilicon column (0.25 mm × 25 m) was used. Temp. 200°C (2 min hold to 380°C (10°C/min). Arrows indicate paeak positions of standard triglycerols. A: trilaurin B: trimyristin C: 1-palmito-dimyristin D: tripalmitin E: 2-oleo-dipalmitin F: tristearin.

TABLE 5. COMPOSITION OF MAJOR FRACTION OF *H. lataniae* CUTICULAR WAX

Group	Composition (%)	Triglycerol (TG)		Identification
I	3.74	2-myristo-dilaurin	MW 666	*m/z* 467 (M-$C_{11}H_{23}COO$), *m/z* 439 (M-$C_{13}H_{27}COO$)
II	18.50	1-lauro-dimyristin (major compound)	M^+ 694 (FD)	*m/z* 495 (M-$C_{11}H_{23}COO$), *m/z* 467 (M-$C_{13}H_{27}COO$)
III	31.62	1-lauro-2-palmito-3-myristin (major compound) and trimyristin	MW 722	*m/z* 523 (M-$C_{11}H_{23}COO$), *m/z* 495 (M-$C_{13}H_{27}COO$) *m/z* 467 (M-$C_{15}H_{31}COO$)
IV	24.18	1-lauro-2-palmito-3-olein, 1-oleo-dimyristin	MW 748	*m/z* 549 (M-$C_{11}H_{23}COO$), *m/z* 521 (M-$C_{13}H_{27}COO$) *m/z* 493 (M-$C_{15}H_{31}COO$), *m/z* 495 (M-$C_{15}H_{29}COO$)
		1-lauro-dipalmitin, 1-palmito-dimyristin	M^+ 750 (FD)	*m/z* 551 (M-$C_{11}H_{23}COO$), *m/z* 523 (M-$C_{13}H_{27}COO$) *m/z* 495 (M-$C_{15}H_{31}COO$)
V	13.09	mixed TG	M^+ 774 (FD)	*m/z* 183 ($C_{11}H_{23}C{\equiv}O^+$), *m/z* 211 ($C_{13}H_{27}C{\equiv}O^+$)
			MW 778	*m/z* 183 ($C_{11}H_{23}C{\equiv}O^+$), *m/z* 211($C_{13}H_{27}C{\equiv}O^+$) *m/z* 239 ($C_{15}H_{31}C{\equiv}O^+$), *m/z* 267 ($C_{17}H_{35}C{\equiv}O^+$)
VI	5.61	tripalmitin and mixed TG	MW 806	*m/z* 467 (M-$C_{17}H_{35}COO$), *m/z* 495 (M-$C_{15}H_{31}COO$) *m/z* 523 (M-$C_{13}H_{27}COO$)
VII	2.11	mixed TG	MW 834	*m/z* 495 (M-$C_{17}H_{35}COO$), *m/z* 523 (M-$C_{15}H_{31}COO$) *m/z* 523 (M-$C_{13}H_{27}COO$)
VIII	0.75	mixed TG	MW 862	
IX	0.26	tristearin and mixed TG	MW 890	

latania triglycerols were also high in stimulant activity (Table 3). Other characteristic constituents were obtained from the eluate with a 1 : 1 mixture of hexane and benzene on a silica gel column: they were identified as α-and δ-amyrin acetates (H. Ageta, personel communication) and shown to be inactive in oviposition stimulation.

DISCUSSION

Our bioassay (Takahashi and Takabayashi, 1984; Takabayashi and Takahashi, 1985) of the effect of oviposition stimulants in the cuticular wax of *Ceroplastes rubens* on females of *Anicetus beneficus* Ishii et Yasumatsu showed that those stimulants were only effective when impregnated on the surface of a paraffin model made in a shape similar to *C. rubens*. Analysis of the oviposition behavior and cuticular wax composition of *A. yanonensis*, in contrast, revealed that the parasitoid has a strong chemical sense by which it recognizes an oviposition site. The strong antennal flagellation behavior on a possible coccoid host regardless of its shape or size indicates that contact chemical sense is an essential cue to induce ovipositional behavior. This insect responded to *U. yanonensis* as well as *U. euonymi* and other coccoids with a similar cuticular wax composition. Although ovipositional response to intact latania scales was less than with *U. yanonensis*, *A. yanonensis* did oviposit on this scale, and offspring emerged. This oviposition on the latania scale was due presumably to a similar stimulant in the cuticular wax. However, the stimulative substance in the latania scale wax was found to be mixed triglycerols: thus *A. yanonensis* may have a strong perception of the ester group in cuticular waxes.

We have investigated the potential host range of *A. yanonensis* for coccoids other than *U. yanonensis* in the laboratory, but further confirmation of the host range in colonized areas is needed. *A. yanonensis* and *C. fulvus*, both introduced from China, have already shown that they will be effective in controlling arrowhead scale populations in citrus orchards in Japan in the near future.

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REARING RATS IN A GERM-FREE ENVIRONMENT ELIMINATES THEIR ODORS OF INDIVIDUALITY

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Abstract—In order to test the hypothesis that commensal bacteria influence the urinary odors of individuality, we collected urine from PVG and PVG.R1 male rats born by cesarian section and reared in a germ-free environment. Using the habituation–dishabituation test with PVG.*RT1*^{u} and Lister hooded rats as subjects, we found that urine from the germ-free rats was not discriminated, while urine from conventionally housed rats of the same strains could be discriminated (experiment 1). When the germ-free rats were moved to a conventional animal house after recolonization with commensural flora and their urine collected, it was discriminated, indicating an essential role of bacteria in determining the unique urinary odors of MHC-congenic rats (experiment 2). The conventionally housed and germ-free rats did not differ in the amount of class I antigen in their urine (experiment 3). Finally, urines of PVG and PVG.R1 donors inoculated with a defined and highly restricted flora to render them specific-pathogen-free (SPF) could not be discriminated. Urine from SPF donors moved to a conventional animal house could be discriminated (experiment 4). These results indicate that commensal bacteria are essential for the production of the unique individual odor of the urine of MHC-congenic strains of rats.

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Key Words—Individual recognition, body odor, odor discrimination, rats, olfaction, major histocompatibility complex, urine, congenic strains, bacteria, rearing conditions.

INTRODUCTION

Animals can identify individuals on the basis of the unique odor of their urine (Brown, 1979; Halpin, 1986), and these individuality signals are important in regulating social behaviors such as mate selection (Yamazaki et al., 1976). Individual odors are also important in the stimulation of physiological "primer" effects of chemosignals, such as pregnancy block, under conditions that provide postconception opportunities for outbreeding (Yamazaki et al., 1983). A genetic basis for the individuality signals in the urine odors of mice and rats has been located to the highly polymorphic transplantation antigens of the major histocompatibility complex (MHC) (Singh et al., 1987, 1988a,b; Yamazaki et al., 1976).

The MHC of the rat (termed the RT1 complex), located on chromosome 14 (Oikawa et al., 1984), influences the unique urinary odors of rats (Singh et al., 1987). The RT1 complex has three different regions: A, B/D, and C/E (Butcher and Howard, 1986). We have shown that males of PVG.*RT1*^{u}, Wistar, Lister, and Long-Evans strains of rat are able to discriminate between urines from rats that differ in all three regions of the MHC (PVG vs. PVG-*RT1*^{av1} donors); only in the classical class I A region (PVG vs. PVG.R1 donors); only in the class I C/E region (PVG.R19 vs. PVG-*RT1*^{av1} donors); only in the class II B/D region (PVG.R1 vs. PVG.R19 donors); and in all regions except the classical class IA locus (PVG-*RT1*^{av1} vs. PVG.R1 donors) (Brown et al., 1987, 1989, 1990; Singh et al., 1987, 1988a,b).

A number of questions can be asked about the distinctive urinary odors that provide the basis for discrimination among MHC-congenic rat strains. We would like to know, for example, what chemicals enable the urines to be discriminated and what physiological mechanism produces these chemicals. We originally thought that the active components of the urine might be the class I molecules themselves, but this is not the case, as the class I molecules purified from the urine of PVG and PVG.R1 males cannot be discriminated, while the urine from PVG and PVG.R1 males with the class I molecules removed by affinity chromatography can still be discriminated (Brown et al., 1987).

We know that the critical chemicals are volatile because we removed the volatile components from the urines of PVG and PVG.R1 males by bubbling inert nitrogen gas through these urines for 48 hr. Test subjects could not discriminate between urines that had the volatile components removed in this fashion (Singh et al., 1988b). The urinary odors of individuality may be due to

small volatile fragments of the class I molecule or to volatile secondary metabolites that are associated with the class I molecules or fragments of these molecules. The volatile components in the urine appear to be physically associated with a protein of molecular weight between 10 and 43 kD (Singh et al., 1987, 1988a,b).

Alternatively, it is possible that each rat has a completely unique bacterial flora and its individuality odor is influenced by products of this bacterial population. It has been suggested that the immune response (*Ir*) gene profile of an individual, determined by the alleles at both the classical class I and class II loci of the MHC, results in the survival of a population of commensal bacterial flora specific to that individual, which produce the volatiles responsible for the MHC-related odor (Howard, 1977). Although there is evidence that can be interpreted as contrary to the bacterial theory (see Brown et al., 1989), this hypothesis has never been directly tested. The present experiments were designed, therefore, to examine the role of bacterial flora in producing the distinctive urinary odor in MHC congenic rat strains.

METHODS AND MATERIALS

Experiment 1

In this experiment we compared the ability of test rats to discriminate between the urines of male PVG and PVG.R1 donors from both conventional and germ-free colonies.

Subjects. The subjects were 66 male PVG-*RT1*^{u} rats born in the specific-pathogen-free (SPF) unit at Babraham and housed in a conventional animal room in groups of three to five in 23.2 × 35.2 × 18-cm plastic cages with wood shavings for bedding. These rats were kept on a reversed 12:12 light–dark cycle with lights off from 8 AM to 8 PM and received ad libitum tap water and food (Labsure CRM NUTS). They were tested when about 180 days of age and all tests were carried out in the dark phase of the light–dark cycle under dim white light illumination.

Conventional Urine Donors. Urine was collected in individual Urimax metabolism cages from four PVG (P) and four PVG.R1 (R) males that were about 180 days of age. These donors were born in the Babraham SPF unit and moved into a conventional animal house when about 120 days of age. After collection, 5-ml pools were made from equal quantities of urine from the four males of each strain, filtered through Whatman No. 1 filter paper, and immediately frozen at −20°C until used in the tests. Urine donors had food (Labsure CRM NUTS) and water available ad libitum throughout the experiment.

Germ-free Urine Donors. Four male PVG and four male PVG.R1 males born by Cesarian section to mothers from the SPF unit, Babraham, and reared

in germ-free isolators (Pendry, 1984) at the MRC unit, Carshalton, served as urine donors. These rats were housed in standard sterile plastic boxes in groups of four within the isolators and had irradiated sterilized water and food (Special Diet Services irradiated RM3 cubes) available ad libitum.

These rats were checked for bacterial sterility by nasopharyngeal swab at 140 days of age and found to be germ-free. Beginning when they were 147 days old, urine was collected daily by placing the rats in sterilized Urimax metabolism cages within the germ-free isolators. The urine collected from individual rats was placed into vials and frozen at −20°C until used for testing.

Apparatus and Procedure. Habituation–dishabituation tests were conducted in a test arena made from a 29.6 × 23.6 × 14.6-cm opaque animal housing cage with a wire-mesh top extending up another 5.6 cm, making the cage 20.2 cm tall (Brown et al., 1987). Odors were presented by placing 0.1 ml of the test liquid onto a 7-cm circle of filter paper (Whatman No. 1) and taping this to one side of the cage so that the center of the filter paper was about 13.5 cm from the floor. A stopwatch was used to record the time that the subjects spent rearing and sniffing at the filter paper. Each odor presentation lasted 2 min, after which time the top of the cage was removed and replaced with another top containing a new piece of odorized filter paper. The used top was then washed with a 70% alcohol solution and allowed to dry before being used again.

Each subject received nine 2-min odor presentations, the first three presentations with water on the filter paper, followed by three presentations of urine sample 1 and three with urine sample 2. Twenty-five subjects were tested with urine samples from conventionally housed donors of the two strains, 12 in the order P–R and 13 in the reverse order. Another 41 subjects were tested with two samples of urine from the germ-free donors, 21 in the order P–R and 20 in the reverse order. Urine from different pairs of donors was used in each test with the germ-free urine.

Statistical Analysis. A randomized blocks ANOVA was performed over the nine urine presentation trials for each group of subjects and Newman-Keuls post-hoc tests were used to investigate differences among the means following a significant overall F test (Kirk, 1968).

Experiment 2

To confirm the results of experiment one, we repeated the experiment with another strain of test subjects. In addition, to verify that these results were due entirely to the germ-free status of the original urine donors, we moved these urine donors to a conventional animal house in order to allow their colonization with normal bacterial flora and then collected urine from these conventionalized donors.

Subjects. Thirty-two male Lister hooded rats about 100 days of age served as subjects. These males had been born in the SPF unit at Babraham and were housed in a conventional animal house in groups of three to six for a week before testing began. We tested eight males with urine from conventionally housed PVG and PVG.R1 rats (four in the order P–R and four in the reverse order). Another eight males were tested with urine from the males reared in germ-free conditions (four in each test order), and 16 were tested with the urine from the germ-free rats that had been moved to a conventional animal house (eight in each test order).

Conventionally Housed Urine Donors. Four PVG and four PVG.R1 males about 90 days of age were moved from the SPF unit at Babraham to a conventional animal house for 10 days. Their urine was collected in Urimax metabolism cages, made into two pools, and frozen at −20°C as described in experiment 1.

Germ-free Urine Donors. Urine from germ-free males was taken from the same batch of samples used in experiment 1.

Conventionalized (Germ-free → Normal) Urine Donors. The same four PVG and PVG.R1 rats that were reared germ-free were gradually conventionalized at Carshalton. They were first given the standard "cocktail" of bacterial culture in their water. This bacterial cocktail is used to produce SPF animals and consisted of: α - hemolytic *Streptococcus* (homionous); *E. coli* (human origin); *Streptococcus bovis* (group D); and *Lactobaccillis acidophilus*. Inoculation with these organisms was undertaken to prevent lethal infection by harmful bacteria, which can occur when animals are conventionalized directly from germ-free conditions (T. Pendry, personal communication, July 1988).

After receiving this cocktail for 24 hr, the rats were moved from the germ-free to an SPF environment for seven days and then housed in a conventional animal house for 48 hr. Urine was then collected for the next 48 hr in Urimax metabolism cages. The urine was made into two pools and frozen at −20°C until used for testing.

Experiment 3

It is possible that germ-free rats differ from conventionally housed rats in the amount of class I molecule excreted in the urine. To test this possibility, we compared urine samples from conventionally housed and germ-free PVG and PVG.R1 rats using enzyme immunoassays.

Enzyme immunoassays (EIAs) were carried out in rigid, nonsterile, flat-bottomed, 96-well polystyrene plates (Nunc Immunoplates II, Gibco Ltd., Middlesex, U.K.); 200 μl of a 20 μg/ml solution of purified anti-class I monoclonal antibody in phosphate-buffered saline (PBS)–0.1% azide, pH 7.4, was used to coat individual wells. The plates were covered and kept at 4°C until use. On

the day of use, excess antibody was removed and wells were postcoated up to the brim with PBS–azide containing 10% v/v fetal calf serum (FCS) for 1 hr at 4°C to block nonspecific protein adsorption sites. After 1 hr the plates were washed twice with PBS + 0.5% w/v Tween 20 (PBST) (Sigma, Poole, England); 200 μl of a solution containing the class I MHC antigen (or a titration thereof) was then added to the appropriate wells and incubated overnight at 4°C. After three washes with PBST and three washes with PBST + 2% v/v FCS, 200 μl of a second noncompetitive anti-class I monoclonal antibody coupled to biotin was added at 8 μg/ml and incubated for 1 hr. The plate was again washed three times with PBST and three times with PBST + 2% v/v FCS before addition of 200 μl of a 4 μg/ml concentration of Streptavidin-coupled horseradish peroxidase (Miles Laboratories, Slough, U.K.). The plates were then incubated for 20 min at 4°C. After washing twice with PBST + 2% v/v FCS and twice under tap water for about 15 sec each time, 200 μl of the substrate 3,3′,5,5′-tetramethyl benzidine at 100 μg/ml was added. The reaction was stopped after 5 min with 50 μl of 2 M H_2SO_4, and the plates were read in a Titertek Multiscan ELISA plate reader (Flow Laboratories Ltd., Irvine, Scotland).

In the assay for detection of the A^{avl} class I MHC antigens of the DA strain of rats (PVG.$RT1^{avl}$), the capture antibody on the plate was JY3/109 (haplotype specificity: *avl*+, *b*+, *c*−, *f*−, *h*−, *l*−, *o*+, *u*−), while the second biotin-labeled antibody was JY1/116 (haplotype specificity: *avl*+, *b*+, *c*−, *f*−, *h*−, *l*−, *n*−, *o*−, *u*−) (Diamond et al., 1984). For the detection of the A^c antigens of the PVG strain, the capture antibody was YR5/310 (haplotype specificity: *avl*−, *c*+, *f*−, *h*+, *l*−, *n*−, *o*−, *u*−), and the biotin-labeled second stage antibody was YR5/12 (haplotype specificity: *avl*−, *c*+, *f*−, *k*−, *l*−, *n*+, *o*−, *u*−) (Maryanski et al., 1986). All are rat IgG alloantibodies.

Experiment 4

Germ-free rats are reared in the complete absence of bacteria, so the germ-free PVG and PVG.R1 donors in experiments 1 and 2 were equal in their absence of bacteria and their urines could not be discriminated. What bacteria are necessary to produce discriminable urinary odors in the two strains? It is possible that the presence of any bacteria would result in the production of discriminable odors. SPF rats of the PVG and PVG.R1 strains, as derived in Babraham, have a defined population of nonpathogenic and aerobic commensal bacteria. Thus, SPF rats are equal in the type of bacteria they initially possess. If the mere presence of bacteria is sufficient for the production of discriminable odors, the urines of SPF donors should be discriminable as are those of conventionally housed rats.

Subjects. Sixteen male PVG.$RT1^u$ and 16 male Lister rats about 140 days

of age served as subjects. These males had been born in the SPF unit at Babraham and were housed in a conventional animal house in groups of three to six as described in experiment 1.

SPF Urine Donors. At Babraham, the SPF rats are derived from germ-free rats by giving them the standard cocktail of bacteria described in experiment 2. Thus, all of these SPF rats have the same commensal bacteria. Urine was collected from four male PVG and four male PVG.R1 rats housed in plastic film isolators (Pendry, 1984) in the SPF unit, Babraham. These rats were maintained on a 12 : 12 light–dark cycle and had irradiated sterilized water and food (irradiated Labsure CRM NUTS) available ad libitum. Urine was collected by placing the donors in individual Urimax metabolism cages inside the isolators. The urine collected from individual rats each day was placed into vials and frozen at −20°C until used for testing.

Conventionalized SPF Urine Donors. The same four PVG and PVG.R1 rats that served as SPF urine donors were moved to a conventional animal house for 10 days. Urine was then collected for the next 48 hr, made into two pools, filtered and frozen at −20°C until used.

Apparatus and Procedure. Habituation–dishabituation tests were conducted as described in experiment 1. Sixteen male PVG.*RT1*^{u} subjects and eight Lister rats were tested with urine from SPF PVG and PVG.R1 donors, half tested in the order P–R and half in the reverse order. The other eight Lister rats were tested with urines from conventionalized SPF PVG and PVG.R1 donors.

RESULTS AND DISCUSSION

Experiment 1

Subjects tested with urine from conventionally housed donors showed significant differences in investigation time over the nine trials for both test orders [P–R: $F(8,88) = 40.97$, $P < 0.001$; *R–P:* $F(8,96) = 26.95$, $P < 0.001$; Figure 1a] and for both test orders, the mean times spent sniffing on trials 4 and 7 were greater than on any of the other trials ($P < 0.01$). The increased time spent investigating the first urine sample (trial 4) indicates that the urines were discriminated from water, and the dishabituation when the second urine sample was presented (trial 7) indicates the ability to discriminate between the two urines.

When the urine donors were germ-free males, there was also a significant difference in time spent sniffing over the nine trials for both test orders [P–R: $F(8,160) = 37.76$, $P < 0.001$; R–P: $F(8,152) = 46.75$, $P < 0.001$]. In both of these cases, the time spent sniffing on trial 4 was greater than that for all other trials ($P < 0.01$), indicating that the urines could be discriminated from water. In neither test order, however, did the time sniffing on trial 7 differ from

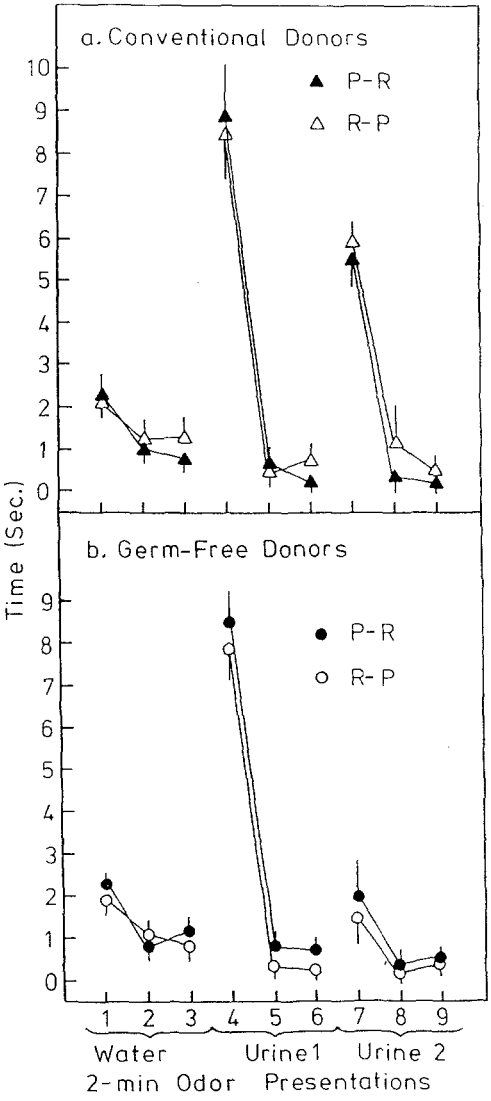

FIG. 1. Mean time (sec ± SEM) spent by PVG.*RT1*^{u} subjects investigating urine odors from (a) conventionally housed PVG (P) and PVG.R1 (R) male rats; and (b) male PVG and PVG.R1 rats reared in a germ-free environment.

the time on trials 5 and 6, indicating that the urines from the two strains were not discriminated (Figure 1b).

The results using urine from conventionally housed rats replicate our previous studies (Brown et al., 1987; 1989, 1990; Singh et al., 1987, 1988a) show-

ing that PVG and PVG.R1 males can be discriminated by their urine odors. The results using urine from germ-free rats indicate that these two strains of rats, when born into and raised in a germ-free environment, cannot be discriminated by their urinary odors.

Experiment 2

As shown in Figure 2a, there was a significant difference in the time spent investigating urines from conventionally housed donors over the nine trials [$F(8, 56) = 28.96$, $P < 0.001$] with more time spent sniffing on trials 4 and 7 than any other trials ($P < 0.01$). Figure 2a also shows that while there was a difference in sniffing times over all nine trials with urine from the germ-free donors [$F(8, 56) = 12.12$, $P < 0.001$), only the time on trial 4 was different from the other trials ($P < 0.01$). These results thus replicate those of experiment one and confirm that bacteria are essential for the production of individual odors.

When the urine was from the conventionalized germ-free rats (Figure 2b), there was a significant difference in sniffing time over the nine trials for both test orders [P–R: $F(8,56) = 26.33$, $P < 0.01$; R–P: $F(8,56) = 17.17$, $P < 0.001$) and for both test orders the times spent sniffing the odor on trials 4 and 7 were significantly different from the other trials ($P < 0.01$). Thus, moving germ-free rats to a conventional environment restores the components of the urinary odor which are used to discriminate between the two strains.

Experiment 3

The enzyme immunoassay (Figure 3) shows that the titer of RT1.A^c class I MHC molecules in the urine of conventionally housed and germ-free PVG rats is identical. The concentration of RT1.A^{av1} molecules in the urine of germ-free PVG.R1 rats also is identical to that found in the urine of conventional PVG.R1 donors. Thus, rearing conditions have had no effect on the production and urinary excretion of soluble MHC antigens. The level of A^c molecules in the urine of normal PVG × DA F_1 hybrids, which carry hemizygous amounts of the A^c and A^{av1} molecules, is intermediate between the levels of the congenic PVG and PVG.R1 strains. This variation in class I levels in urine parallels the natural variation in the levels of expression of the membrane-bound class I molecules in the tissues of these strains (Howard et al., 1979) and is not a special feature of the soluble or excreted forms of these molecules.

Although we have previously shown that the class I molecule itself does not impart the individuality signal to the urine (Brown et al., 1987), it is possible that different quantities of class I molecule in the circulation or in the urine may be associated with differences in urinary odor. The results of this experiment indicate that differences in the urine odors of germ-free and conventionally housed rats are not due to differences in the quantity of class I antigens in the urine.

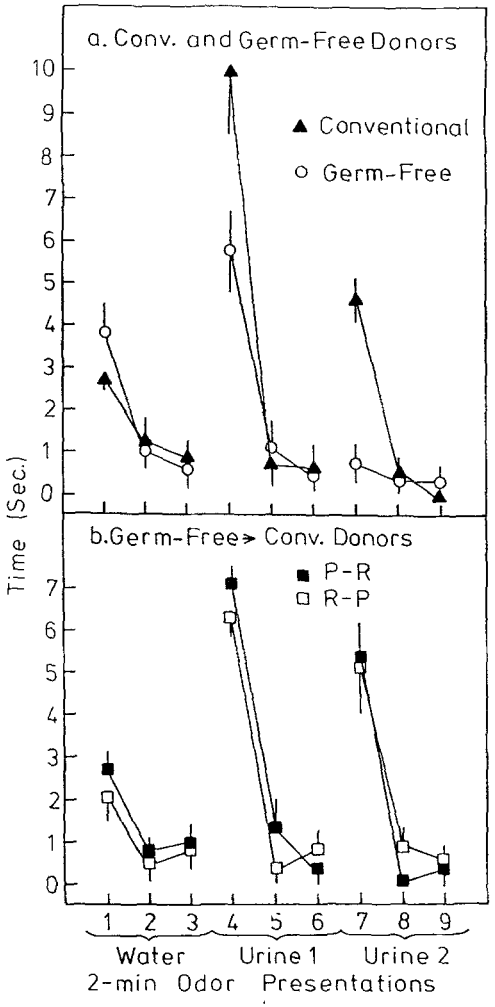

FIG. 2. Mean time (sec ± SEM) spent by Lister hooded subjects investigating urine odors from (a) conventionally housed and germ-free PVG and PVG.R1 male rats; and (b) male PVG and PVG.R1 rats reared in a germ-free environment and then moved to a conventional environment before urine collection.

Experiment 4

The PVG.*RT1*^{u} subjects showed significant differences in time sniffing odors over the nine presentations [$F(8,120) = 40.64$, $P < 0.001$ for both orders of presentation combined]. The time spent sniffing on trial 4 was significantly

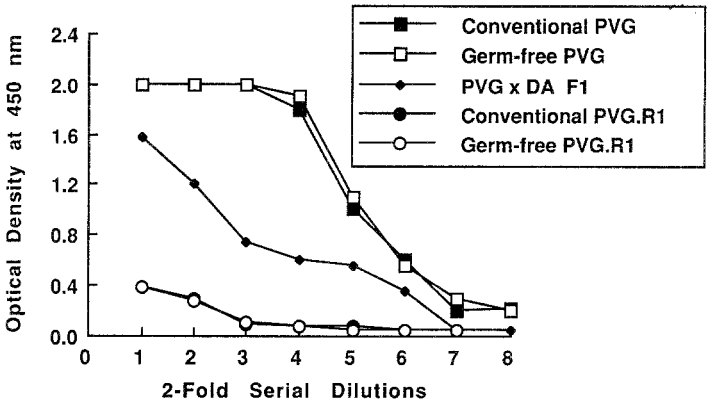

FIG. 3. Two-site enzyme immunoassay of soluble classical class I antigens in urine from germ-free and conventional PVG (RT1.A^c) and PVG.R1 (RT1.A^{av1}) rats. The level of A^c molecules in the urine of normal PVG × DA F_1 hybrids is also shown.

greater than on any other trial ($P < 0.01$), but the time on trial 7 did not differ from that on trials 5 and 6 (Figure 4a). Likewise, the Lister subjects showed a significant difference in time spent investigating the odors over the nine trials [$F(8,56) = 17.80$, $P < 0.001$ for both orders of odor presentation combined] and only the time spent sniffing on trial 4 differed from the other trials ($P < 0.01$). (Figure 4a).

When the urine was from donors that had been taken from the SPF unit and housed in a conventional animal house, the Lister subjects showed a significant difference in the investigation times over all nine trials [$F(8,56) = 26.51$, $P < 0.001$, for both test orders combined] and the time spent sniffing on both trials 4 and 7 were greater than on all other trials ($P < 0.01$), indicating that the two urine odors could be discriminated (Figure 4b).

Thus, the urine of congenic strains of rats housed in the SPF unit could not be discriminated, while the urines of SPF donors moved to a conventional animal house could be discriminated. The bacteria necessary to produce the odors of individuality in MHC congenic rats, therefore, are not present in the SPF rats but are acquired once these rats are placed in a conventional animal house. Furthermore, it is possible to use the cheaper and more readily available SPF rats rather than germ-free animals to determine which organisms are necessary to produce the urinary odors of individuality. SPF rats can be inoculated with families comprising many different strains of bacteria, and if their urine is discriminable, inoculation with individual bacteria from these families can be undertaken to reveal the strain(s) required to produce the MHC-specific odors that enable discrimination to occur.

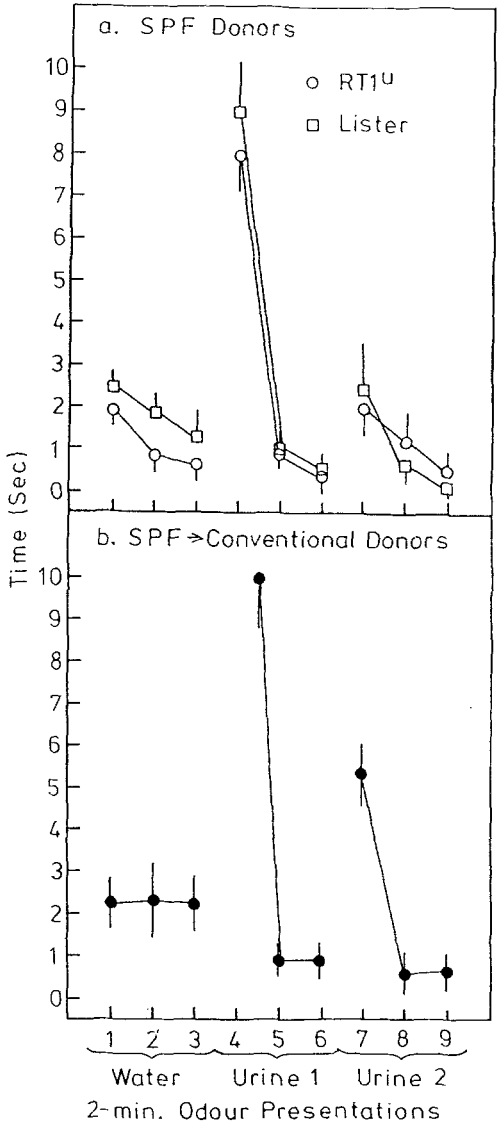

FIG. 4. Mean time (sec ± SEM) spent by (a) PVG.*RT1*^{u} and Lister subjects investigating urine odors from specific-pathogen-free PVG vs. PVG.R1 donors, and (b) Lister subjects investigating urine odors from conventionalized SPF donors.

General Discussion

We examined urine from germ-free donors to test the hypothesis that the influence of the MHC was indirect and involved control of the urinary odor through immune response (*Ir*) genes. These genes, which map to the MHC, regulate the number and/or type of bacteria that inhabit the gut, skin, and urinary tract (Howard, 1977; Hamilton, quoted in Crozier, 1987). Many instances of bacterial influences on the production of social odors in mammals have been documented (Brown, 1979; Albone, 1984), and it is known that the urinary profiles of germ-free and conventional rats differ in many volatile and acidic metabolites (Holland et al., 1983).

The finding that urine from germ-free donors contained no individuality signals linked to the MHC (Figures 1b and 2a), despite the presence of the class I antigens in the urine (Figure 3), although predicted by the *Ir* gene theory, was surprisingly clear-cut. The necessary role for bacteria in producing the MHC-specific odor was confirmed using two different strains of test rats and by moving the germ-free rats in stages to a conventional animal house whereupon the ability of test animals to discriminate between urine samples collected from the previously germ-free donors was restored (Figure 2b).

Essentially, this result is compatible with two types of mechanism. First, the urinary excretion of the MHC class I transplantation antigens (Brown et al., 1989; Singh et al., 1987, 1988a) is a mischievous coincidence, and their undoubted functional role as individuality markers when attached to cell membranes is unrelated to any role as individuality markers in the urine. In this case, the individuality odor of the urine would be independently determined by commensal flora unique to each individual and regulated by *Ir* genes.

Alternatively, the excretion of class I molecules does have an essential role in determining the individual odor of the urine. Bacteria might be considered an essential source of a body pool of odorant molecules that do not vary among individuals. The class I molecule selects a cocktail of odorants from this body pool and, acting as a carrier molecule, delivers it to the urine in a manner analogous to the way in which the MHC binds and presents immunogenic peptides to the immune system (Bjorkman et al., 1987a,b).

If the former hypothesis is correct, we are left with the remarkable fact that every individual in a population must carry a unique and stable bacterial flora, the products of which regulate the crucial breeding strategies that determine the genetic constitution of the population. If, however, the second "carrier hypothesis" is correct, it might offer a simple, inherently stable, genetic mechanism by which a common and potentially variable bacterial population can contribute vitally to the individual odor of mammals.

The latter hypothesis also can accommodate a role for the class II region of the MHC in body odor (Brown et al., 1989; Yamazaki et al., 1990). It is

into this region of the MHC that the classical *Ir* genes map. The variation in the ability of specific class II molecules in selecting individual antigens for presentation to the immune system is the molecular mechanism by which they act as immune response genes (Klein, 1986). Thus, there can be an overriding role of class II in determining body odors through broad variations in bacterial flora. The class I molecules could then select individual-specific odorant profiles.

Two observations that are more consistent with the second hypothesis than the first are that the odor of serum from congenic strains, although attractive to test animals, is not discriminated, and thus carries no individuality signal, while urine from these same individuals is readily discriminated (Brown et al., 1987a; Yamazaki et al., unpublished). If the MHC-specific odor is due to a unique set of odorants in the body pool produced by unique bacterial flora, it is difficult to see why serum should not express the individuality signal. Second, fractionation of urine by dialysis (Singh et al., 1988b) and gel filtration (G. Beauchamp, personal communication, November 1988) has shown that the active fraction that can be used by test animals to discriminate between MHC types is associated with a high-molecular-weight component of the urine. This favors a carrier role for the soluble class I MHC molecules in determining the MHC specific odor profile (Singh et al., 1988a).

The MHC of the rat and mouse are comparable, and differences in homologous regions of the rat and mouse MHC result in discriminably different urine odors in both species (Brown et al., 1990; Yamaguchi et al., 1981; Yamazaki et al., 1982, 1990). We would predict, therefore, that bacteria may control the production of MHC-specific odors in mice and other species as they do in rats.

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EFFECT OF FUNGAL METABOLITE PERAMINE AND ANALOGS ON FEEDING AND DEVELOPMENT OF ARGENTINE STEM WEEVIL (*Listronotus bonariensis*)

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Abstract—Peramine, a pyrrolopyrazine alkaloid produced by the fungal endophyte of perennial ryegrass *Acremonium lolii*, deterred the feeding of both adults and larvae of the graminacious herbivore, the Argentine stem weevil (*Listronotus bonariensis*), at 0.1 μg/g and 10 μg/g, respectively. In a no-choice test fewer stem weevil larvae fed and developed on diet containing as little as 2 μg/g peramine. The proportion of larvae which did not develop beyond the first instar was higher on diet containing peramine and appeared to be due to a higher proportion of larvae which did not feed. For larvae which fed on the peramine-containing diet, feeding scores and times to pupation were not significantly different from those of controls. A number of simple peramine analogues showed feeding-deterrent activity against adult weevils, indicating the importance of the pyrrolopyrazine ring system of peramine in determining feeding-deterrent activity.

Key Words—Perennial ryegrass, *Lolium perenne*, Gramineae, Argentine stem weevil, *Listronotus bonariensis*, Coleoptera, Curculionidae, *Acremonium lolii*, endophyte, feeding deterrent, peramine.

INTRODUCTION

The resistance of perennial ryegrass (*Lolium perenne* L.) to the Argentine stem weevil [*Listronotus bonariensis* (Kuschel), Coleoptera: Curculionidae], which arises from the presence of the endophytic fungus *Acremonium lolii* Latch,

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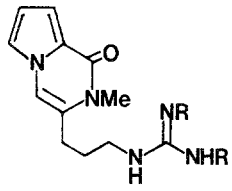
Christensen and Samuels, was first reported by Prestidge et al. in 1982. Since then, examples of the insect resistance of grasses arising from the presence of endophytic fungi have been reported for an increasing number of grass and insect species (Clay et al., 1985; Clay, 1989; Hardy et al., 1985; Gaynor and Rowan, 1986; Siegel et al., 1985, 1987). Siegel et al. (1987) list seven species from four insect orders that are deterred from feeding on grasses infected with *A. lolii*, eight species from four orders deterred by *A. coenophalium* in tall fescue [*Festuca arundinacaea* (Schreber)] and three species from two orders deterred by *Ephichloe typhina* in grasses. More recently the southern armyworm *Spodoptera eridania* (Ahmad et al., 1987) and the pasture mealy bug (*Balanococcus poae*) (Hemiptera: Pseudococcidae) (Pearson, 1988) also have been shown to be affected by the presence of *A. lolii* in ryegrass.

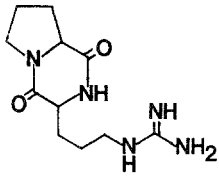
The Argentine stem weevil is a major pest of ryegrass pastures and of maize, wheat, barley, and brassica crops in New Zealand (Power, 1984; Prestidge et al., 1985). Adult Argentine stem weevil produce windowlike grazing scars on leaves but generally cause little permanent damage to established pastures. The larvae are more destructive. Eggs laid on ryegrass leaf sheath tissue hatch to produce tunneling larvae that burrow into the middle of the grass tiller, chewing first the innermost rolled leaf and growing point and then the base of the outer leaves. Larvae may transfer from tiller to tiller, mining and killing from three to five tillers as they develop through their four instars.

Endophyte-infected ryegrasses suffer less feeding damage by adult weevils and have fewer eggs and larvae than do uninfected plants (Gaynor and Hunt, 1983; Barker et al., 1983, 1984a,b). When given a choice between clonal material infected or uninfected with *A. lolii*, adult stem weevils show more feeding on the uninfected leaves (Gaynor et al., 1983). Extracts from endophyte-infected leaves were shown to contain an extractable feeding deterrent, peramine, which was purified by bioassay-guided fractionation (Rowan and Gaynor, 1986) and identified as the novel guanidinium alkaloid, 3-(3′-guanidinylpropyl)-2-methylpyrrolo[1,2-a]pyrazin-1(2H)-one (Scheme 1, **1a**) (Rowan et al., 1986). Peramine contains two novel structural features: A reduced diketopiperazine heterocyclic ring system and a strongly basic guanidinium group, neither of which have previously been reported in insect feeding deterrents.

A second group of endophyte-associated metabolites, the lolitrem neurotoxins, have also been isolated from endophyte-infected perennial ryegrass and are considered responsible for the neuromuscular syndrome of livestock known as ryegrass staggers (Gallagher et al., 1981, 1984). Lolitrem B at 5 μg/g was not a feeding deterrent to adult stem weevils in choice tests but did cause increased mortality and reduced the growth of larval weevils (Prestidge and Gallagher, 1985, 1988). Typical concentrations of 1–7 μg/g dry weight of lolitrem B in endophyte-infected pasture have been reported (Gallagher et al., 1985; Prestidge and Gallagher, 1988). While lolitrem B was considered a pos-



1a R=H
1b R=Ac



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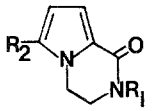
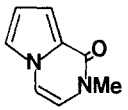


3a R_1=H, R_2=H
3b R_1=Me, R_2=H
3c R_1=Me, R_2=Br

SCHEME 1.

sible cause of resistance to Argentine stem weevil larvae, the deterrence of adult feeding and its associated egg-laying was considered a more important mechanism of resistance (Prestidge and Gallagher, 1985).

Information on the resistance factors produced by endophyte-infected plants and their effects on the behavior and development of target insects is necessary for the effective use of endophyte-mediated insect resistance. A more complete study of the biological activity of peramine and an evaluation of its role in the stem weevil resistance of endophyte-infected ryegrass has been hindered by the limited quantities of peramine available from herbage. Recently, peramine was identified in the seeds of perennial ryegrass and a more efficient isolation procedure based on ion-exchange chromatography was developed (Rowan and Tapper, 1988). This procedure yields crystalline salts of peramine that have been used to test the activity of peramine against both adult and larval Argentine



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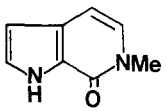

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SCHEME 2.

stem weevil. Synthetic approaches to the previously unknown 2-methylpyrrolo[1,2-a]pyrazin-1(2H)-one ring system of peramine (Brimble et al., 1988) have also yielded a series of analogs to test structure–activity relationships between the heterocyclic ring system and the guanidinyl functions. These studies form the basis of this paper.

METHODS AND MATERIALS

Peramine as the sulfate and hydrobromide salts was obtained from seeds of perennial ryegrass (Rowan and Tapper, 1988). Diacetylperamine (**1b**) was synthesized from peramine by acetylation with acetic anhydride in pyridine at room temperature for 6 hr. Evaporation of the reagents under reduced pressure and preparative thin-layer chromatography on silica gel with 25% acetone in dichloromethane gave diacetylperamine (**1b**), which was recrystallized from 1,2-dichloroethane/heptane, mp 141–142°C (Rowan et al., 1986). Diacetylperamine was tested against adult stem weevil at concentrations of 0.1, 1, and 10 μg/g. Cycloprolylarginine (**2**) was synthesized from prolyl-*N*ω-nitroarginine benzyl ester, after cyclization using ammonia in methanol and removal of the nitro protecting group using palladium on carbon, and was a gift from Dr. M.T. Brimble (DSIR, Palmerston North). Ion-exchange chromatography on Dowex 2 × 8 (chloride form) gave cycloprolylarginine hydrochloride (**2**). FAB-MS; 254($M + H^+$); [^{1}H]NMR (80 MHz, CD_3OD, dioxane internal standard) δ 1.88 (8H, m), 3.15 (2H, t, J = 6Hz), 3.43 (2H, t, J = 6.7 Hz), 4.23 (2H, br. d, J = 4 Hz); [^{13}C]NMR (20 MHz, CD_3OD, dioxane internal standard) δ 23.1 (t), 24.5 (t), 27.2 (t), 29.0 (t), 42.0 (t), 46.3 (t), 55.7 (d), 60.1 (d), 158.1 (s), 167.9 (s), 173.0 (s). Cycloprolylarginine hydrochloride (**2**) was tested at concentrations of 10, 32 and 100 μg/g. The analogs **3a–c**, **4**, and **5** (Scheme 2)

were obtained by synthesis (Brimble et al., 1988) and tested against adult stem weevil at concentrations of 1 and 10 μg/g. Subsequently compounds **3a** and **3b** were retested at 32 μg/g diet.

Adult Stem Weevil Feeding Choice Bioassay

Field-collected adult Argentine stem weevils were conditioned to a 4% agar, 4% cellulose powder, 5% sucrose diet for several days and starved for 24 hr before each bioassay. Feeding choice experiments were performed using the method of Rowan and Gaynor (1986), except that three weevils were used per 9-cm-diam. Petri dish. Each dish contained two plugs of agar diet; one containing the test substance and the other a control. Assays were randomized, and feeding was scored blind after 72 hr at 16°C, 16:8 hr light–dark on a scale of 0–3 (0, no feeding; 1, one small area of disk broken up by feeding; 2, two to three areas broken up; 3, more than three areas shredded). Thirty-six or 37 replicates were used for each concentration tested. The difference in feeding scores between the two agar disks of each Petri dish was analyzed using the Wilcoxon signed-rank test (Siegel, 1956). The mean difference between the feeding scores of weevils on the test and on the control disks (MDFS) was calculated as a measure of the feeding preference of the insects.

Larval Stem Weevil Feeding Bioassay

Larval stem weevil were obtained from eggs laid by captive adult weevils on endophyte-free ryegrass plants. Eggs were surface sterilized for 5 min in 1% formaldehyde in 70% ethanol with 0.01% Tween 80 added as a wetting agent.

No-Choice Bioassay. Single eggs were transferred on moist filter paper to 2-ml clear plastic analyzer cups (11.5 mm ID, Salmond-Smith Biolab). Each cup contained a 0.5-ml plug (10 mm diam.) of wheat-germ-based diet (Malone and Wigley, 1990) cut with a corkborer to fit the internal dimensions of the cup. Peramine hydrobromide in 80% methanol–water was adsorbed onto the cellulose powder used to make up the diet so as to give final concentrations of 0, 2, 10, and 25 μg peramine hydrobromide/g of diet. Potassium bromide was adsorbed onto the cellulose powder added to the diets to give a bromide concentration in all diets of 6.1 μg/g. Larvae were maintained at 18°C and 16:8 hr light–dark. Feeding was scored on a 0–3 scale (as for the adult weevils) every three or four days, and the head capsule widths of all larvae that were suitably positioned against the wall of the container were measured at the same time with an eyepiece micrometer to determine the stage of larval development. Head capsule widths of first-instar larvae ranged from 0.138 to 0.215 mm (micrometer settings 6–9); second instars from 0.227 to 0.305 mm (micrometer 10–13); third instars from 0.317 to 0.440 mm (micrometer 14–19); and fourth instars from 0.455 to 0.630 mm (micrometer 20–24). In some instances where a larva was not visible, the larval instar was unambiguously deduced from preceding

and subsequent observations. For each peramine concentration, 72 replicates were used. The arrangement of the analyzer cups was randomized. Feeding scores and head capsule widths were measured blind.

When about to pupate, larvae were transferred to 2-ml analyzer cups containing moist vermiculite and were checked daily to see if pupation had occurred. The number of larvae feeding, the feeding scores, and the number of larvae pupating were analyzed using a SAS categorical model (SAS, 1985). The effect of peramine on the proportion of first-instar larvae was analyzed using the chi-square test. The effect of peramine on the number of days to pupation was analyzed by ANOVA.

Choice Bioassays. Single eggs were transferred on moist filter paper to each of 42 2-ml analyzer cups containing two 0.5-ml plugs of diet. One plug of diet was prepared using cellulose powder to which sufficient peramine hydrobromide had been adsorbed to give a final concentration of 10 μg peramine hydrobromide/g diet. The other plug of diet was prepared after treating the cellulose powder with the appropriate solvent (solvent control). The control and test diet plugs were separated by a 1.2-cm length of 28-gauge nichrome wire between each diet plug. The position of the two diet plugs relative to the lid was randomized; one egg was placed between the plugs; and the cups were laid horizontally to remove any gravitational effects. Feeding on each of the diet plugs was scored blind on a 0–3 scale as above after 28 days and analyzed by a Wilcoxon signed-rank test.

RESULTS

Effect of Peramine on Feeding Choice

Adult weevils in choice tests showed significantly less feeding on diet containing as little as 0.1 μg/g peramine hydrobromide (Table 1). Peramine hydrobromide at 0.01 μg/g had no effect on weevil feeding, suggesting a minimum inhibitory concentration of peramine for adult weevils of between 0.01 and 0.1 μg/g.

Stem weevil larvae, presented with peramine hydrobromide in a choice test at the single concentration of 10 μg/g, showed significantly less feeding on the peramine containing diet (Table 1).

Effect of Peramine on Larvae

In a no-choice situation, peramine hydrobromide significantly reduced the number of larvae that fed on the diet and that reached pupation (Table 2). The effect of peramine was dose dependent, and significantly fewer larvae fed and reached pupation even at the lowest concentration of 2 μg peramine hydrobrom-

TABLE 1. EFFECT OF PERAMINE ON FEEDING OF ARGENTINE STEM WEEVIL ADULTS AND LARVAE

	Peramine sulfate concentration (μg/g)	Feeding preference (number of insects)				*P* (Wilcoxon signed-rank test)
		Prefer peramine	Avoid peramine	No preference	No feeding	
Adults	0.01	9	13	11	5	0.20
	0.1	4	23	6	5	0.001
	1.0	2	27	3	5	0.001
	1.0	4	30	0	8	0.001
Larvae[a]	10	2	25	8	0	0.001

[a] Scored after 28 days.

ide/g of diet. Larval mortality was higher on all diets containing peramine. For those larvae that did feed on the diet, mean feeding scores and times to reach pupation were not significantly different from those of controls (Table 3). Survival to eclosion did not appear to be related to peramine concentration.

Head capsule widths of the larvae were measured to determine the rate of larval development. Head capsule data recorded after 32 days were excluded because of the low numbers of larvae observed (13–44%) and the onset of pupation. Consistently more larvae were observed on the diets containing peramine, reflecting a decreased tendency for larvae to burrow and feed on this diet. As insufficient numbers of second-, third-, and fourth-instar larvae were observed for separate analysis, the numbers of larvae at these instars were combined, and the data were analyzed in terms of the effect of peramine on the percentage of

TABLE 2. EFFECT OF PERAMINE ON FEEDING AND DEVELOPMENT OF LARVAL ARGENTINE STEM WEEVIL

Concentration peramine hydrobromide (μg/g)	Number hatched	Larvae feeding (%)[a]	Entering pupation (%)	Mortality (%)[a]
0	68	77.9	50.0	7.4
2	50	42.0	40.0	16.0
10	54	35.2	35.2	16.7
25	55	23.6	18.2	20.0
P		<0.01[b]	<0.01	

[a] Scored after 32 days.
[b] Probability that differences between sequential values in each column are due to chance.

TABLE 3. EFFECT OF PERAMINE ON STEM WEEVIL LARVAE THAT FED ON PERAMINE-CONTAINING DIET

Concentration peramine hydrobromide (μg/g)	Number feeding[a]	Mean feeding score	Number surviving to eclosion	Mean days to pupation (±SEM)
0	53	2.85b[b]	13	37.6 ± 1.7c
2	21	2.90b	7	37.9 ± 4.2c
10	19	2.47b	5	40.4 ± 3.8c
25	13	2.54b	6	39.0 ± 3.4c

[a] Scored at 32 days.
[b] b,c: Means followed by the same number are not significantly different.

first-instar larvae (Figure 1). The use of only two categories in the data analysis enabled the inclusion of data for second- or later instar larvae that were not observed again before pupation. Peramine significantly ($P < 0.02$) increased the proportion of first-instar larvae, compared to controls, from day 11 of the experiment. A higher percentage of first-instar larvae were observed at higher peramine concentrations, and this increase in the number of first-instar larvae remained highly significant ($P \leq 0.001$) until day 32.

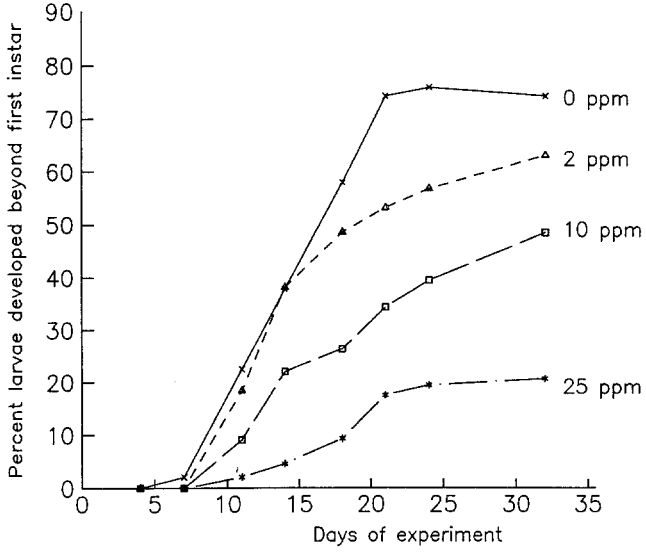

FIG. 1. Effect of peramine on the larval development of the argentine stem weevil.

Feeding-Deterrent Activity of Peramine Analogs

The feeding deterrent activity of peramine hydrobromide and analogs is summarized in Table 4. Acetylation of peramine gave the nonbasic, lipophilic diacetyl derivative **1b**, which was active as a feeding deterrent to adult stem weevil at 10 ($P < 0.001$) and 1 ($P = 0.03$) but not 0.1 μg/g ($P = 0.2$). While the feeding-deterrent activity of **1b** suggested that the basic guanidinyl group was not required for feeding-deterrent activity, this interpretation is complicated by the possibility that diacetylperamine (**1b**) was partially hydrolyzed to peramine (**1a**) during the preparation of the agar for the bioassay. This possibility was not investigated further, rather cycloprolylarginine (**2**) was tested as a more appropriate peramine analog. Cycloprolylarginine (**2**) proved inactive as a feeding deterrent at 100 μg/g ($P = 0.28$), indicating that the specific pyrrolopyrazinone ring system rather than the propylguanidinyl side chain was important for feeding-deterrent activity.

Chemical synthesis (Brimble et al., 1988) gave the simple heterocyclic lactam (**3a**), which proved inactive as a feeding deterrent at 32 μg/g ($P = 0.22$). Methylation of lactam (**3a**) gave the *N*-methyl lactam (**3b**), which showed feeding-deterrent activity at 32 ($P = 0.004$) and 10 ($P = 0.006$) but not at 1 μg/g ($P = 0.28$). The bromo-lactam (**3c**) and unsaturated lactam (**4**) were also active as feeding deterrents at 10 μg/g ($P < 0.05$) but not 1 μg/g. The structural isomer **5** was inactive as a feeding deterrent at 10 μg/g (MDFS = −0.29, $P = 0.22$).

TABLE 4. FEEDING DETERRENT ACTIVITY OF PERAMINE AND ANALOGS AGAINST ADULT STEM WEEVIL AS SHOWN BY MEAN DIFFERENCE IN FEEDING SCORES (MDFS) RECORDED ON TEST AND CONTROL DIETS

	Concentration (μg/g)					
Compound	0.01	0.1	1.0	10	32	100
Peramine (1a)	0.21[a]	1.16***	1.77***			
1b		−0.27	0.78*	2.05***		
2				0.32	0.88*	0.32
3a				−0.24	0.21	
3b			0.19	0.69**	1.44***	
3c			−0.09	0.88**	1.57***	
4			0.17	0.86**		

[a]Data from Table 1; *significantly less feeding ($P < 0.05$) on the test diet; **significantly less feeding ($P < 0.01$) on the test diet, ***significantly less feeding ($P < 0.001$) on the test diet.

DISCUSSION

Peramine, a pyrrolopyrazine alkaloid produced by the endophyte of perennial ryegrass, has been shown to be a feeding deterrent to both adult and larval Argentine stem weevil. Fewer stem weevil larvae fed or developed to pupation on diets containing peramine. Peramine significantly reduced the mean head capsule widths of larvae on diet containing peramine (data not shown); however, this reduction in mean development did not represent a delayed development for all larvae. Rather, as the concentration of peramine increased, a higher proportion of larvae showed no evidence of feeding and remained at the first instar of development.

At each of the concentrations of peramine tested, some larvae did feed on the peramine-containing diet. These larvae appeared to develop normally and showed feeding, development times, and survival comparable to controls. While larval mortality was higher on diet containing peramine, this may be related to the reduced number of insects feeding rather than to any direct toxicity. Individual larvae appeared to either feed or not feed, with a higher proportion showing no feeding at higher peramine concentrations. Peramine appears to function solely as a feeding deterrent at the concentrations tested. Somewhat similar responses have been reported for all the fall armyworm *Spodoptera frugiperda* presented with the feeding deterrent, aristolochic acid (Raffa and Frazier, 1988). While the onset of feeding was delayed by the presence of aristolochic acid, once larvae had begun to feed, the rate of leaf consumption was not significantly different from that of larvae feeding on control tissue.

As an insect feeding deterrent, peramine possesses a unique combination of lipophilic heterocycle and strongly basic guanidinium functionalities. Feeding-deterrent assays using a number of peramine analogs indicated the importance of the heterocyclic pyrrolopyrazine ring system rather than the propylguanidinyl side chain for feeding-deterrent activity against adult Argentine stem weevils. The inactivity as a feeding deterrent of the simple lactam (**3a**), together with the feeding-deterrent activity of its methylated analog (**3b**) suggests minimal but precise structural requirements for feeding-deterrent activity. All active analogs were, however, less active as feeding deterrents than peramine itself, suggesting some importance for the guanidinyl side chain in obtaining the full biological response.

Peramine has been shown now to be a feeding deterrent to larval and adult Argentine stem weevils. Stem weevil larvae transfer between grass tillers, and the neonates, reared on an artificial diet, wander for some time before burrowing into the diet plug (Power and Singh, 1974). We have observed similar behavior, especially on diet containing peramine where feeding deterrence may be inferred. This suggests that the presence of peramine may cause the larval Argentine stem weevil to reject endophyte-infected plants. Concentrations of

peramine in the range of 5–47 μg/g dry weight occur in samples of perennial ryegrass infected with *A. lolii* (Tapper et al., 1988). These concentrations are comparable to those at which peramine shows activity against the stem weevil and suggest feeding deterrence of both adult and larval weevils may contribute to the lower level of stem weevil damage observed on endophyte-infected ryegrass and tall fescue (Barker et al., 1983; Gaynor and Hunt, 1983).

A number of other insect species are reported to be affected by the presence of *A. lolii* in perennial ryegrass (Siegel et al., 1987; Ahmad et al., 1987; Pearson, 1988), but to date these insects have not been tested against peramine. Peramine is also present in tall fescue infected with *A. coenophalium*, in ryegrasses and fescues infected with *Epichloe typhina* (Siegel and Rowan, unpublished results), and in seedlings of annual ryegrasses infected with an unnamed *Acremonium*-like endophyte (Dymock et al., 1988). Annual ryegrass seedlings infected with endophyte show resistance to Argentine stem weevil (Stewart, 1985), but this resistance disappears as the plants mature and as peramine levels drop to below 1 ppm (Dymock et al., 1988). Endophyte-infected tall fescue is resistant to Argentine stem weevil (Barker et al., 1983), but contains, in addition to peramine, endophyte-related ergopeptine (Bacon et al., 1986) and loline alkaloids (Johnson et al., 1985). The role of peramine in the insect resistance of other endophyte-grass associations remains to be determined.

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INGESTION OF TALL LARKSPUR BY CATTLE
Separating Effects of Flavor from Postingestive Consequences[1]

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Abstract—Tall larkspur (*Delphinium* spp.) is a palatable but toxic poisonous plant in the western United States. The toxins in tall larkspur are diterpenoid alkaloids. We examined the influences of food flavor and postingestive consequences on consumption of a 33% larkspur pellet during 30-min feeding periods for five days using esophageally fistulated cattle that were sham-fed larkspur pellets. Consumption by the sham-fed group was compared to a control group fed alfalfa pellets, and a larkspur group fed only larkspur pellets. Sham-fed cattle did not decrease ($P > 0.1$) feed consumption compared to controls, indicating no significant difference in food flavor. The larkspur group decreased ($P < 0.05$) feed consumption by 41% relative to controls and by 31% relative to sham-fed animals ($P = 0.08$). This reduction in feed consumption indicates the adverse postingestive consequences of tall larkspur ingestion, as the larkspur group apparently developed a conditioned taste aversion to the larkspur pellet. Even though these animals were averted to the pellets, they showed none of the classical signs of intoxication from ingestion of tall larkspur.

Key Words—Feeding behavior, alkaloids, palatability, conditioned taste aversion, cattle, poisonous plants, tall larkspur, *Delphinium barbeyi*.

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INTRODUCTION

Tall larkspur (*Delphinium* spp.) causes major livestock losses in the western United States and Canada (Ralphs et al., 1988). Larkspur contains toxic levels of diterpenoid alkaloids and is most toxic early in the growing season (Olsen, 1983). Alkaloid concentrations are inversely correlated with larkspur ingestion. Early in the growing season, when alkaloid levels can be as high as 3–6% of dry weight, intake of larkspur is low (Pfister et al., 1988a,b). However, cattle consume much higher levels of mature larkspur, which contains 0.2–1.5% total alkaloids (Pfister et al., 1988a,b).

There is little information concerning the factors that influence the consumption of tall larkspur by grazing animals (Ralphs et al., 1988; Pfister et al., 1988a,b). Livestock supposedly reject tall larkspur as forage because of the bitter taste of alkaloids (Bate-Smith, 1972; Ralphs and Olsen, 1987; Ralphs et al., 1988). However, livestock may also learn to reduce consumption due to adverse postingestive impacts of alkaloids (Provenza et al., 1990). Although many toxic compounds do not produce conditioned food aversions (Garcia et al., 1985; Provenza et al., 1988), the malaise induced by larkspur extracts or lithium chloride causes cattle to form aversions to larkspur (Olsen and Ralphs, 1986; Lane et al., 1990).

Pavlov pioneered the use of sham-fed animals to separate the effects of taste from nutritional consequences (see Boakes, 1984). Sham-feeding involves the use of esophageally fistulated animals so feed is diverted from the digestive tract, thus avoiding gastrointestinal consequences (Grovum and Chapman, 1988). This study employed this technique to evaluate the influences of larkspur flavor and postingestive effects on consumption of larkspur pellets by cattle.

METHODS AND MATERIALS

Plant Collection. Tall larkspur (*Delphinium barbeyi*) was collected during August 1986 from a central Utah mountain range at 3200 m elevation. Most larkspur plants were in the pod stage, when larkspur is not very toxic; at this stage of maturity, grazing cattle consume about 20% of their diet as tall larkspur (Pfister et al., 1988a). Larkspur was air-dried, ground through a 2-mm screen, and stored in plastic bags at room temperature. Larkspur was mixed 1:2 with ground alfalfa hay and pelleted using minimum heat to form a 33% larkspur pellet. Commercial alfalfa pellets served as the control feed. The source for the alfalfa was the same for the larkspur and alfalfa pellets. The larkspur pellet was analyzed for total diterpenoid alkaloids by the procedure of Manners and Ralphs (1989). *D. barbeyi* contains 17 individual alkaloids (Pelletier et al., 1989); the

toxic alkaloid methyllycaconitine is present in *D. barbeyi*, but the toxicity of this and other alkaloids from *D. barbeyi* is unknown (Olsen and Manners, 1989).

Bioassay. The bioassay involved three groups, each containing four steers. Before the trial, all animals were adapted for one week to alfalfa pellets. Group 1 (control; body weight 333 ± 23 kg) consisted of steers that received only alfalfa pellets during days 1–12. After an overnight fast, these animals received alfalfa pellets ad libitum daily for 3.5 hr from 0800 to 1130 hr. Consumption was measured at 30 min and again at the end of the period. This allowed animals sufficient time to consume pellets in amounts above 2% of body weight, a level slightly above maintenance for growing steers (NRC, 1976).

The second group (larkspur group) was used to assess the combined effects of flavor and postingestive consequences associated with larkspur. The four steers in this group (332 ± 34 kg) received the same amounts of alfalfa pellets as group 1 during days 1–4 (preliminary period). On days 5–7, the steers were given larkspur pellets for the first 30 min and then were given ad libitum access to alfalfa pellets for 3 hr. This procedure familiarized animals with the larkspur pellets, a novel food (Provenza et al., 1990). Results of a previous study (Pfister et al., 1989) suggested that cattle would not consume enough 33% larkspur pellets during this 30-min period for three days to cause any adverse effects. On days 8–12, these cattle received only larkspur pellets for 3.5 hr, and consumption was measured as before.

Group 3 (sham-fed group; 404 ± 22 kg) consisted of steers fitted with established esophageal and ruminal fistulae to separate the effects of flavor from any postingestive influences. Feed ingested by these esophageally fistulated animals is extruded from the esophagus (Grovum and Chapman, 1988) and does not reach the rumen. Before the trial, we established via ruminal evacuation that essentially all feed ingested when the esophageal plug was removed was extruded during a 30-min feeding period. Esophageally fistulated animals are usually fed this long because the fistula tends to constrict and impair sample collection after about 30–40 min (Pfister et al., 1990). Furthermore, the duration was restricted to 30 min to avoid confounding from lack of satiety in sham-fed animals (Baile and McLaughlin, 1987). On days 1–4, these animals were fed alfalfa pellets, and consumption was monitored as before with the esophageal fistula plug in place. From days 5 to 12, the esophageal plug was removed for the first 30 min to measure the ingestion of larkspur pellets. The plugs were then replaced, all masticated larkspur feed was removed from the floor of the pen, and animals were allowed to eat alfalfa pellets ad libitum for 3 hr. Days 5–7 were used to familiarize the animals with the larkspur feed, and feed intake was monitored during days 8–12.

Statistical Analysis. Feed intake during the 30-min period and subsequent 3-hr period was analyzed using SAS (1987), as a repeated measures analysis of

variance with individual steers ($N = 4$) nested within treatment groups ($N = 3$), with repeated measurements during days 5–7 and days 8–12 (Gill, 1978). The Huynh-Feldt assumption (SAS, 1987) required for a repeated measures analysis was satisfied. When significant treatment effects were found, the LSD procedure was used to separate means. Because of size differences among groups of animals, amounts consumed were adjusted for body weight (g/kg body weight).

RESULTS

Cattle in the control group ($P = 0.04$) and in the sham-fed group ($P = 0.08$) consumed more pellets during the 30-min period (days 8–12) than did the larkspur group (Table 1, Figure 1). The larkspur group consumed 59% and 69% of the amount eaten by the controls and sham-fed group, respectively. The sham-fed group consumed 85% ($P > 0.1$) of the amount eaten by the control group during the 30-min period. There was a day effect ($P < 0.05$), and no day by treatment interaction ($P = 0.1$).

The sham-fed group ate more pellets ($P < 0.05$) during the 3-hr period than the control group (Table 1, Figure 2), which consumed more pellets ($P < 0.05$) than the larkspur group. There was no day effect ($P = 0.11$), and no day by treatment interaction ($P = 0.24$). Actual feed ingested by the sham-fed group (3 hr during trial) rose about 6% over the pretrial level (3.5 hr). Conversely, the larkspur group decreased total feed intake about 43% compared to pretrial

TABLE 1. CONSUMPTION OF ALFALFA AND 33% LARKSPUR PELLETS (g/kg BODY WEIGHT) DURING 30-MIN PERIOD AND SUBSEQUENT 3-hr PERIOD BY CATTLE

	30-min intake			3-hr intake		
Means[a]	Control	Larkspur	Sham-fed	Control	Larkspur	Sham-fed
Days 1–4	8.8	9.7	8.3	14.1	14.2	13.4
Days 5–7[b]	7.5	5.4	6.9	16.2c	10.7c	23.9d
Days 8–12	8.0c[c]	4.7d	6.8c	16.0c	8.9d	22.9e

[a] No day by treatment interactions, therefore only main effects are presented. All animals were fed alfalfa pellets during the preliminary period, days 1–4. Controls were fed alfalfa pellets during days 5–12. Larkspur and sham-fed groups were given larkspur pellets for a 30-min period and alfalfa pellets for a 3-hr period during days 5–7 and days 5–12, respectively. Larkspur group received only larkspur pellets during days 8–12.

[b] No treatment effect on 30-min intake ($P > 0.1$).

[c] c, d, e: Means for each variable within a period followed by a common letter do not differ ($P > 0.08$).

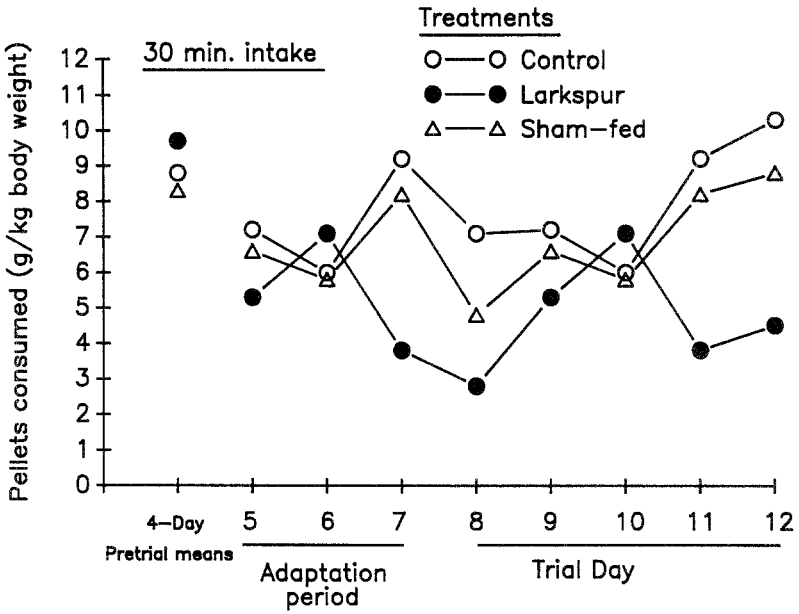

FIG. 1. Ingestion of pellets (g/kg body weight) by control, larkspur, and sham-fed steers during 30-min periods. Days 1–4 were a preliminary period when all animals were fed alfalfa hay pellets; only the four-day mean is shown. Days 5–7 were an adaptation period to familiarize animals with novel larkspur pellets.

levels. Total intake of alfalfa pellets by the control group increased 4% during days 8–12 compared to their pretrial level.

The total alkaloid concentration of the larkspur pellets was 0.48%.

DISCUSSION

This study indicates that larkspur flavor did not inhibit animal feeding, as the sham-fed group consumed similar amounts of larkspur pellets as controls did of alfalfa pellets. Sham intakes represent primarily flavor factors, because no ingested larkspur reached the rumen of the sham-fed animals, and the feed was not novel. This study confirms previous grazing trials indicating that larkspur flavor is generally acceptable to cattle (Pfister et al., 1988a,b).

The primary impact of larkspur was adverse gastrointestinal consequences acting to reduce ingestion. The sham-fed and larkspur groups showed no initial adverse reaction to the flavor of the larkspur (days 5–6, Figure 1), but by day 7 the larkspur pellets had adversely affected the larkspur group, as consumption

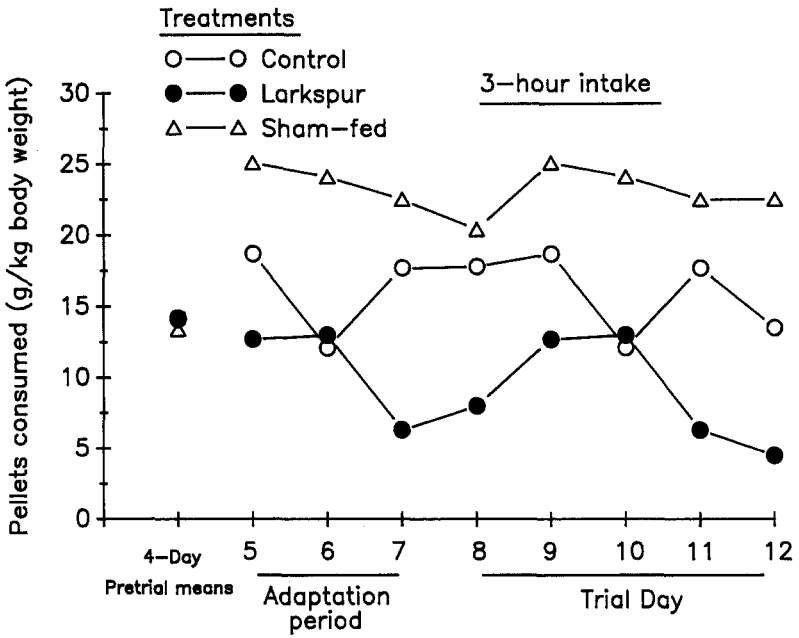

FIG. 2. Ingestion of pellets (g/kg body weight) by control, larkspur, and sham-fed steers during 3-hr feeding periods when allowed ad libitum feed consumption. Days 1–4 were a preliminary period when animals were fed alfalfa hay pellets; only the four-day mean is shown. Days 5–7 were an adaptation period to familiarize the animals with the novel larkspur feed.

fell. Our assumption that consumption of larkspur pellets for 30 min would not cause adverse postingestive consequences was apparently incorrect. During the five-day trial, intake of larkspur pellets by the larkspur group was reduced by 31% compared to the sham-fed group. Tall larkspur apparently induced a conditioned taste aversion (CTA) (Olsen and Ralphs, 1986; Burritt and Provenza, 1989a,b; Lane et al., 1990). CTAs occur when animals associate the taste of the feed with adverse gastrointestinal consequences and reduce feed intake (Garcia et al., 1985). We have no clear indication of the nature of the gastrointestinal malaise in cattle consuming larkspur. Pfister et al. (1989) indicated that sublethal levels of tall larkspur have no adverse effects on digestibility, passage rate, or fermentation when given intraruminally to cattle. Olsen and Ralphs (1986) used larkspur alkaloid extracts to induce CTA to larkspur in cattle, but acute toxicity (Olsen, 1978) was not evident. In our study, the larkspur animals limited their feed intake on days 7 and 8, increased consumption of pellets on days 9 and 10, and reduced consumption on days 11 and 12. Three animals in

the larkspur group apparently suffered from distress by days 7, 8, 11, and 12, and ate very little for two consecutive days; however, we observed none of the classical signs of larkspur intoxication such as muscular tremors or sternal recumbency (Olsen, 1978).

Field studies have shown that individual animals tend to consume larkspur in a two- to four-day cycle, alternating between periods of higher and lower consumption (Pfister, unpublished data 1986), indicating that adverse post-ingestive consequences may be important under grazing conditions. Constant daily doses of alkaloidal extracts have maximum effect at three to four days (J.D. Olsen, unpublished data 1989). Grazing animals may develop short-term aversions to larkspur, but our observations indicate that aversions formed under grazing conditions will extinguish as animals begin to sample larkspur in small amounts with no apparent deleterious consequences (Pfister et al., 1988a; Ralphs, unpublished data 1989). Cattle have a propensity to sample feed items, and we speculate that cattle can often determine the amount of larkspur that can be safely consumed based on postingestive consequences. Sheep will cautiously sample harmful feed mixed with lithium chloride, and limit intake below a level that will cause gastrointestinal distress (Thorhallsdottir et al., 1987; Burritt and Provenza, 1989a). Alternatively, the taste and/or odor stimulus of larkspur may change as plant phenology changes, thus altering the taste and odor cues a naturally averted animal may use to detect and continue avoiding harmful larkspur (Palmerino et al., 1980; Eisner and Grant, 1980).

Our results emphasize the importance of understanding the factors controlling ingestion of larkspur by cattle. Assessing the alkaloidal fractions in larkspur at various phenological stages is warranted and may provide valuable information for future management recommendations by predicting the risk of consumption and intoxication by grazing cattle. Further research is also needed on practical aspects of using CTAs to train cattle to avoid larkspur (Provenza and Balph, 1988; Lane et al., 1990).

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ISOLATION AND CHARACTERIZATION OF PHYTOTOXIC COMPOUNDS FROM ASPARAGUS (*Asparagus officinalis* L.) ROOTS[1]

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Abstract—Potential allelochemicals from aqueous extracts of dried asparagus (*Asparagus officinalis* L.) roots were isolated and characterized. Active fractions separated by HPLC included ferulic, isoferulic, malic, citric, and fumaric acids. Soxhlet extraction of the residues also produced phytotoxic caffeic acid. Although none of these compounds, when applied singly, was active enough to account for the phytotoxicity of asparagus extracts, their combined effect might be additive or synergistic. An extract from lyophilized fresh root tissues contained a fraction that was one order of magnitude more toxic than any compound obtained from the dried roots. The most active component was isolated by TLC and characterized by [^{1}H]NMR as methylenedioxycinnamic acid (MDCA). This compound provided severe inhibition of curly cress (*Lepidium sativum* L.) root and shoot growth at concentrations of 25 ppm or above.

Key Words—Allelopathy, asparagusic acid, autotoxicity, phytotoxicity, caffeic and, citric acid, ferulic acid, fumaric acid, isoferulic acid, malic acid, methylenedioxycinnamic acid, asparagus.

INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is a perennial vegetable grown on sandy soils in temperate areas of the eastern and western United States. "Asparagus decline" reduces yield and quality and ultimately results in death of crowns in

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many production areas (Endo and Burkholder, 1971; Grogan and Kimble, 1959; Hartung et al., 1989; Herner and Vest, 1974). Asparagus decline has been attributed to infection by two persistent soil-borne pathogens, *Fusarium oxysporum* (Schlecht.) emend. Snyd. and Hans. f. sp. *asparagi* Cohen, and *F. moniliforme* (Sheld.) emend, Snyd, and Hans. (Cohen and Heald, 1941; Endo and Burkholder, 1971; Grogan and Kimble, 1959; Johnston et al., 1979).

Other laboratories as well as this one, however, have presented evidence that asparagus is an autotoxic or autoallelopathic plant (Hartung and Stephens, 1983, 1984; Hartung et al., 1989; Laufer and Garrison, 1977; Shafer and Garrison, 1980a, b, 1986; Yang, 1982; Young 1984). Autotoxicities are considered as special cases of allelopathy, although the same compounds may exert interspecies effects. Compounds released from living or senescing asparagus root tissue were hypothesized to damage the asparagus root system, which in turn decreased the growth of the crowns (Lanfer and Garrison, 1977; Shafer and Garrison, 1980a, b, 1986; Yang, 1982; Young, 1984, 1986). Injury to the root system from these autotoxins may also provide infection courts for the entrance of the *Fusarium* pathogens, thereby enhancing the incidence of disease on the plant (Hartung and Stephens, 1984). Young (1986) has reported that several phenolic compounds are released from the root system of intact asparagus plants. Using an XAD-4 resin trapping procedure originally developed to recover phenolic and chlorophenoxy herbicides from soil, he isolated 3,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dimethoxyacetophenone, and *m*-hydroxyphenylpropionic acid. However, he did not report the effect of these compounds on asparagus seedlings nor did he determine if senescing tissue possessed any inhibitory compounds that also might contribute to the decline problem in the field. He also did not determine if these compounds were derived from asparagus specifically or were from other plants or microbes.

Kitahara et al. (1972) isolated three compounds, named growth regulators, from etiolated asparagus ferns, asparagusic acid (1,2-dithiolane-4-carboxylic acid) with activity comparable to abscisic acid, dihydroasparagusic acid (β,β'-dimercaptoisobutyric acid), and (*S*)-acetyldihydroasparagusic acid (β-(*S*)-acetyl-β'-mercaptoisobutyric acid). Although these compounds were extremely inhibitory to seedling germination and radical elongation, they occurred at extremely low concentrations in the tissues and were quite labile once isolated. Asparagusic acid also has been isolated from the roots of asparagus and found to be inhibitory to nematodes, specifically the emergence of the second-stage larvae of *Heterodera rostochiensis* and *H. glycines*, the second-stage larvae of *H. rostochiensis* and *Meloidogyne hapla*, and the larvae and adults of *Pratylenchus penetrans* and *Pratylenchus curvitatus* (Takasug: et al., 1975). A patent is presently held by Sankyo Co. Ltd., Japan, on asparagusic acid and its derivatives for activity on nematodes and plant growth regulation.

Since asparagus possesses large storage roots that are continually dying as the crown grows, large amounts of live, senescing, and dead asparagus root material may be present in fields at any one time. These roots and residues apparently release numerous toxic substances that can inhibit seed germination and seedling growth of different species (Shafer and Garrison, 1980a, b, 1986), as well as asparagus growth, peroxidase activity of asparagus seedlings, and respiration of the crown (Hartung et al., 1989). At this point, no compounds have been identified that can account for all the inhibitory activity exerted by the root tissue. The purpose of this research was to isolate compound(s) from asparagus root tissue responsible for reported allelopathic properties of asparagus and to quantify their toxicity with bioassays.

METHODS AND MATERIALS

Preparation of Dried Root Tissues. Asparagus crowns (Martha Washington) were excavated from a research plot (7 years old) located at the Horticultural Research Farm, Michigan State University, East Lansing, Michigan. The storage and fibrous roots were separated from the rhizomes, and all dead and visible diseased plant material was discarded. The roots were oven dried at 50°C and ground in a Wiley mill (mesh screen size 1 mm). All plant tissues were sterilized with propylene oxide gas and checked for microbial contamination as previously reported (Hartung and Stephens, 1983; Hartung et al., 1989). Containers with sterilized root tissue were allowed to exhaust under a fume hood for 24 hr to dissipate any residual propylene dioxide. Dried sterilized tissue was stored in brown glass bottles at −20°C until used for chemical isolation.

Bioassay Procedure for Evaluation of Isolated Components of Asparagus Root Extractions. Previous studies in this laboratory have shown that curly cress (*Lepidium sativum* L.) is a fast and reliable bioassay for evaluating the inhibitory activity of many allelopathic compounds (Lehle and Putnam, 1982). All fractions to be assayed were first taken to dryness and weighed. Separate fractions were then redissolved in methanol (or an appropriate solvent, depending on solubility of the fraction), and a dilution series was made. A known amount of each fraction was spotted on filter paper (Whatman No. 1) in glass Petri dishes. Evaporated solvent controls and deionized water controls were always included in each bioassay. All fractions were filter sterilized before bioassaying. The solvent was allowed to evaporate completely from the filter paper, and 1.5 ml of deionized H_2O was added to the dish containing 10 curly cress seeds. The dishes were covered, randomized, and placed in a moist chamber for 72 hr at 26°C. Root length was measured and I_{50} levels determined by interpolation. There were three replications per treatment in all cases except when paucity of

the compound only allowed for two replications. The data were subjected to analysis of variance and means compared with the LSD test. When available, standards of isolated compounds were tested for inhibitory activity from 0.025 μg/ml to 2.0 mg/ml.

Isolation of Inhibitory Components from Aqueous Root Extracts. Since previous work (Hartung and Stephens, 1983; Laufer and Garrison, 1977; Shafer and Garrison, 1980b) indicated that aqueous extracts were phytotoxic, they were first subjected to sequential extraction with solvents in an attempt to partition the chemical components. Milled asparagus root tissue was extracted by stirring overnight at 4°C (1:10 root tissue–deionized H_2O). The particulates were removed by filtration and centrifugation at 6000 *g* for 20 min. The proteins and lipids in the supernatant were precipitated with acetone (4:1 v/v acetone–sample) by slowly stirring overnight at 4°C. The precipitate was filtered and bioassayed. Since this fraction showed no activity, it was discarded subsequently and the liquid concentrated to 1/4 the original volume on a rotary evaporator (Buchi) at 40°C. This concentrate was sequentially extracted with the following solvent series: chloroform, diethyl ether, dichloromethane, ethyl acetate, and water-saturated *n*-butanol. The aqueous root extract was partitioned three times (1:1 v/v), with each of the five solvents. Each solvent fraction was dried with $MgSO_4$, concentrated to dryness by rotary evaporation at 40°C, weighed, and bioassayed at 10 and 100 ppm. The percent of the original fraction shown by each solvent extraction was calculated.

The chloroform fraction was further separated by column chromatography (Figure 1). The methanol-soluble fraction was further purified using an octadecyl (C_{18}) bonded phase solid support in a Baker flash chromatography column (190 × 20 mm) eluted with a step gradient of acetonitrile (100%) to methanol (100%) at 25% intermediate steps in 100-ml fractions. Pressure for the column was provided by a laboratory air line at a rate of 0.2 cm/sec. Fractions (25 ml) were collected, spotted on thin-layer chromatography plates (Whatman silica gel 60 F-254; mobile phase: chloroform–methanol 9:1 and chloroform–methanol 8:2), then recombined according to similar *Rf* values. The fractions were dried under nitrogen, weighed, and bioassayed at 25, 50, and 100 ppm. Individual fractions were then further purified by high-pressure liquid chromatography (HPLC) using a C_{18}, 8-mm × 10-cm radial compression column (Waters μBondapak). The UV detector (Waters 490 multiwave programmable) measured absorbance at 254 and 280 nm. The solvent system was acetonitrile–H_2O (1:99) at a flow rate of 1.0 ml/min. The mixture was resolved into five distinct areas, and five fractions were collected after repeated injections. Three separate fractions from the silica gel flash columns were further separated in this manner. Each fraction from the HPLC collection was dried under nitrogen, weighed, and bioassayed at 50 ppm on curly cress. Purified samples were silylated and then analyzed by gas chromatography–mass spectroscopy (GC-MS) using a

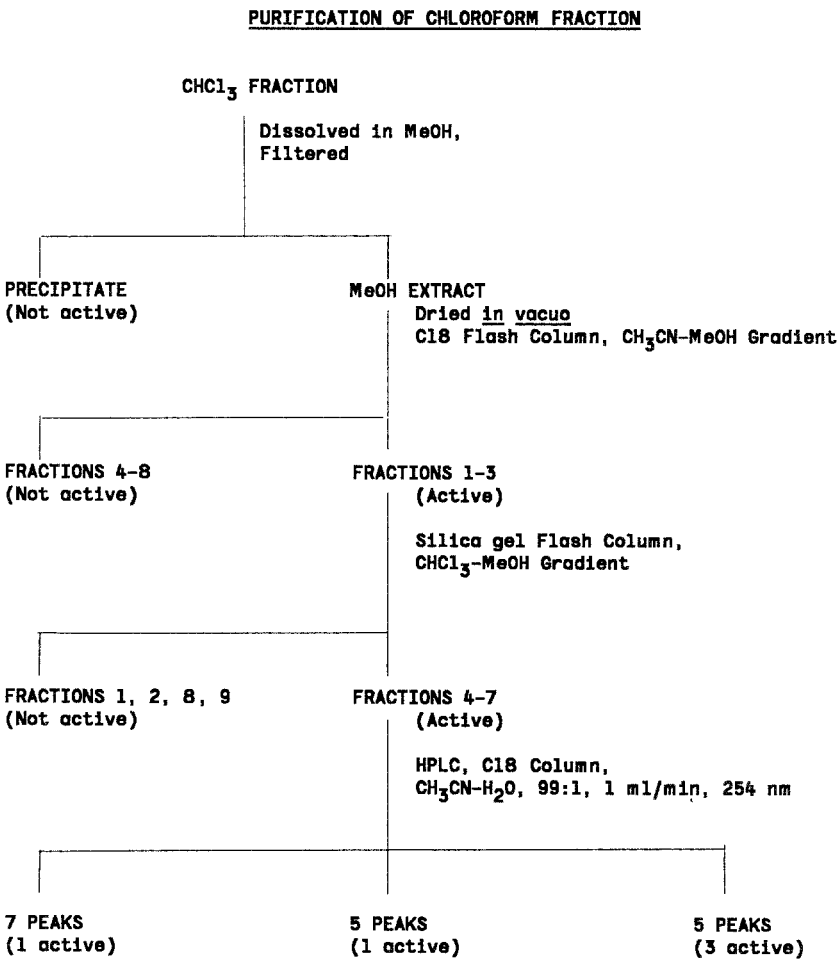


FIG. 1. Chromatographic separation of chloroform extracts from dried asparagus root tissues. A total of five active fractions were obtained from the repetitive HPLC analysis of the initial three fractions collected.

temperature gradient from 110 to 250°C at 15°/min. Spectral scans from total ion chromatograms were observed, and a library search was done on each spectrum using the Finnigan NBS Library Compilation series. Standards of elucidated compounds were then injected into the GC-MS (15°/min on a 30-m DB5 column) under the same conditions to further substantiate the identification of these compounds by matching their GC retention times, as well as comparing mass spectra.

The methanol-soluble portion of the chloroform fraction isolated as above also was separated using preparative TLC. Silica gel (Merck DC-Fertigplatten, 60 F-254 0.5 mm, 20 × 20 cm) plates were first washed in 85 : 15 chloroform–methanol, then approximately 600 mg of the methanol-soluble chloroform fraction was streaked near the base of the plate. Nine distinct bands were scraped off the silica plates and eluted from the silica gel with 100% chloroform, (or chloroform–methanol, 5 : 1 v/v for bands at R_f 0.4), which was passed through a fritted glass filter. The fractions were collected, dried, weighed, and bioassayed at 30 ppm. One fraction (R_f 0.53) was rechromatographed on silica gel plates as previously described. Again, nine fractions were detected by UV (254 nm), scraped, and eluted from the plates as above. Portions of five of these fractions (R_f 0.69, 0.61, 0.52, 0.46, 0.29, and 0.07) were silylated for GC-MS analysis, and the [^{1}H]NMR spectra (deuterated methanol) of the remaining portions of each of these small fractions were obtained on a Varian XL 300-MHz instrument.

Dried asparagus root tissue (250 g) was extracted with hexane for 4 hr using Soxhlet extraction. The hexane was removed and replaced with methanol, and the tissue was further extracted overnight with methanol. The methanol extract then was acidified by dilute HCl to pH 2.0 and extracted with 50 ml of either five times. This ether fraction then was extracted with 75 ml of 5% aqueous sodium bicarbonate two times. The alkaline portion then was acidified with HCl to pH 2.0, then extracted with 50 ml of ether three times. Fractions then were visualized on TLC after development with mobile phases, chloroform–methanol (8.5 : 1.5) and toluene–ethyl formate–formic acid (5 : 4 : 1). In the last ether fraction, a yellow compound crystallized on the sides of a round-bottomed flask. These crystals were removed by decanting the ether from the flask, washing the crystals with hexane, and dissolving the crystals in methanol.

Extraction and Isolation of Compounds from Fresh Tissues. Fresh asparagus roots (2 kg) collected from the Horticulture Research Center, East Lansing, Michigan, were blended with deionized H_2O (2.8 liters) and filtered through double-layered cheesecloth. The viscous water extract upon lyophilization at 5°C (FTS tray lyophillizer) afforded a hygroscopic powder (260 g). This was stirred with $CHCl_3$ (2.6 liters) at room temperature (30 min) and filtered through a sintered glass filter funnel. Evaporation of the $CHCl_3$ extract in vacuo gave a brown gum (180 mg). The $CHCL_3$-insoluble residue was dried and kept in a desiccator. Preliminary bioassay on cress seeds with these fractions indicated higher levels of toxicity in the $CHCl_3$ extract. Hence, the $CHCl_3$ insoluble residue was stored at 0°C and further analysis was carried out on the $CHCl_3$ extract. Ferulic acid, isoferulic acid, and caffeic acid were chromatographed against the $CHCl_3$ extract by TLC (silica, $CHCl_3$–MeOH, 4 : 1, v/v), and the band that did not correspond to the standards used was collected and eluted with $CHCl_3$–MeOH (1 : 1). Removal of the solvent in vacuo gave a col-

orless solid, [^{1}H]NMR (CD_3OD): δ 6.00 (1H, s, OCH_2O), 6.20 (1H, d, J = 15 Hz, olefinic), 7.00 (3H, m, aromatic), 7.55 (1H, d, J = 15 Hz, olefinic), 9.20 (1H, bs, COOH); MS (CI, CH_4), m/z at 193 (100%, M+H).

RESULTS

Extraction of Dried Root Tissue. Water extraction of dried asparagus root tissue gave approximately 35% of the mass from the original material. After acetone precipitation, approximately 83% of the water extract remained. Chloroform extraction accounted for 0.022%, diethyl ether, 0.009%; dichloromethane, 0.004%; ethyl acetate, 0.011%; and *n*-butanol, 0.12% of the original dried root material. When bioassayed on curly cress, the $CHCl_3$ fraction contained the highest specific activity (I_{50} = 17.5 ppm in this extraction) (Figure 2). The diethyl ether fraction also had considerable activity (I_{50} = 34 ppm). Dichloromethane and ethyl acetate fractions also contained inhibitory activity but to a lesser extent than the chloroform and ether fractions (I_{50} = 82 and 100 ppm, respectively). Also, at lower concentrations, the dichloromethane and ethyl acetate fractions were slightly stimulatory. Because the greatest inhibitory activity was in the chloroform fraction, it was chosen for further isolation attempts. By refining the isolation technique, chloroform sometimes removed as much as 0.17% of the original dried material, but based on a weight-for-weight basis, I_{50} levels were always very similar, usually being less than 40 ppm for each isolation.

Fractionation of the methanol-soluble portion of the chloroform fraction on an octadecyl bonded phase solid support flash column and subsequent bioassay showed the first three fractions to be inhibitory (I_{50} = 25 ppm). However, these active fractions still contained several chemical components and were further purified on a silica gel flash column. Nine fractions were collected, four of which gave 98% inhibition of curly cress at less than 50 ppm. I_{50} levels for these fractions were 35, 58, 18, and 30 μg/ml for fractions 4, 5, 6, and 7, respectively.

Purification and Identification. The most active fraction 6 was purified further on HPLC, and the five peaks collected were bioassayed on curly cress. Only one peak area proved to contain inhibitory activity. This component, when evaluated by GC-MS, still appeared to contain about 12 different compounds. A library search done for matching spectra and injection of known standards at the same conditions on the GC-MS revealed the presence of fumaric acid, malic acid, isoferulic acid, ferulic acid, dihydrocitric acid, and citric acid, as well as several sugar moieties (Figure 3). When standards of identified compounds were bioassayed on curly cress, I_{50} levels were 0.5, 1.46, and 1.58 mg/ml for ferulic acid, malic acid, and citric acid, respectively.

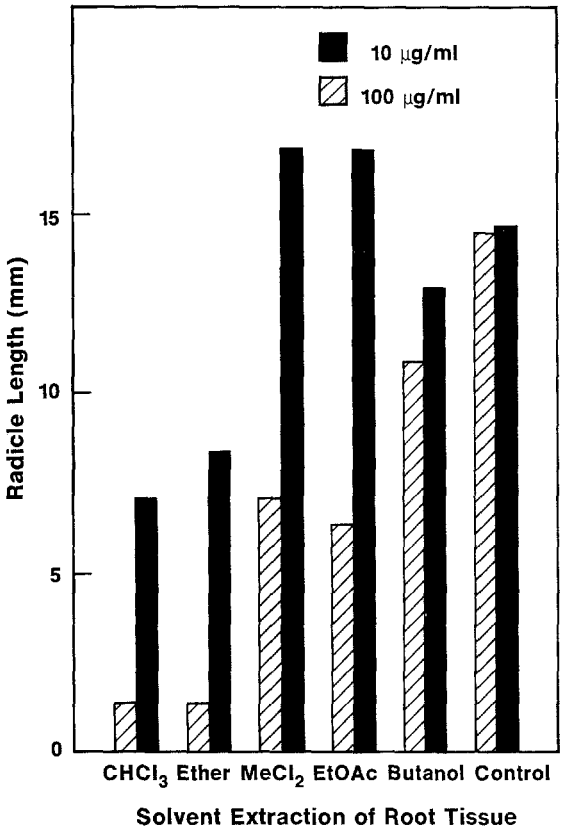


FIG. 2. Inhibition of curly cress root growth by five different organic solvent extracts from dried asparagus root tissue.

The HPLC fraction at R_f 0.07 was subjected to [^{1}H]NMR in CD_3OD. The spectrum revealed signals representing asparagusic acid (1,2-dithiolane-4-carboxylic acid), and the chemical shift values were in agreement with those published. The GC-MS of the silylated derivative of this fraction gave ions at *m/z* 222, 189, 129, and 73. This fragmentation pattern also suggested asparagusic acid.

When a crystallized component from the last ether fraction was subjected to [^{1}H]NMR in CD_3OD, the spectrum revealed signals at δ 7.53 (1 H, d, J = 15 Hz), 7.01 (1 H, d, J = 2 Hz), 6.92 (1 H, dd, J = 8, Hz), 6.78 (1 H, d, J = 8 Hz), and 6.21 (1 H, d, J = 15 Hz). This compound also gave a molecular ion at *m/z* 180 under EI mass spectral conditions and analyzed for $C_9H_8O_4$. These data suggested the structure was that of caffeic acid (2,4-dihydroxycin-

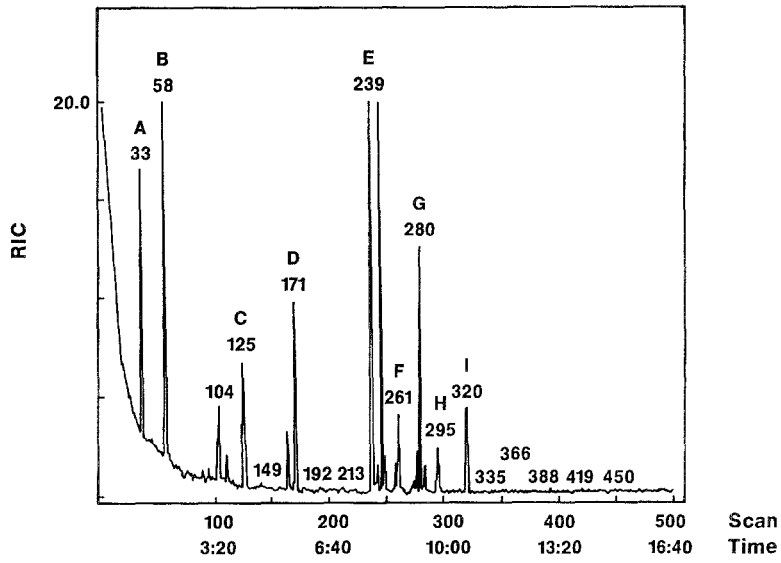
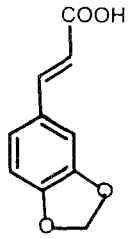


FIG. 3. Total ion current of GC-MS of fraction 3 of peak collected from HPLC separation of dried asparagus root extract that was inhibitory to cress seed germination and radicle elongation. A, B = silylated peaks; C = fumaric acid; D = malic acid; E = dihydrocitric acid; F = mannose; G = isomer of ferulic acid; H = glucopyranose; and I = ferulic acid or isoferulic acid.

namic acid). Standard caffeic acid run under the same [^{1}H]NMR condition produced an identical spectrum.

[^{1}H]NMR and MS analyses of the active compound isolated from fresh tissues indicated that it was methylenedioxycinnamic acid (MDCA) (Figure 4). The spectra were identical to that provided by a commercial sample. This com-



MDCA

FIG. 4. Structure of methylenedioxycinnamic acid (MDCA), a potential allelochemical isolated from fresh asparagus tissue.

TABLE 1. RELATIVE ACTIVITIES OF SEVERAL ORGANIC ACIDS ISOLATED FROM ASPARAGUS ROOTS ON CURLY CRESS ROOT AND SHOOT GROWTH

	Inhibition (%) of root				Inhibition (%) of shoot			
Compound[a]	50 ppm	100 ppm	250 ppm	500 ppm	50 ppm	100 ppm	250 ppm	500 ppm
FA	30	41	50	84	0	0	5	79
IFA	0	0	0	17	0	0	0	5
CA	0	0	0	0	0	0	6	25
MDCA	67	78	79	89	59	77	80	100
MIX	6	17	52	72	0	9	31	98

[a]FA = ferulic acid; IFA = Isoferulic acid; CA = caffeic acid; MDCA = methylenedioxycinnamic acid; MIX = additive amounts of FA, IFA, and MDCA to obtain the respective total concentration studied.

pound was bioassayed with several of the other compounds previously isolated from asparagus roots. MDCA was more inhibitory than any of the other cinnamic acid derivatives tested (Table 1). Even at 50 ppm, it provided more than 50% inhibition of cress root and shoot growth.

DISCUSSION

Isolation of compounds from asparagus root extracts revealed the presence of several known acidic compounds that have been previously reported to be important in allelopathic interactions. Ferulic acid has been reported as a germination inhibitor produced by *Camelina alyssum* and present in residues of corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), sorghum [*Sorghum halepense* (L.) Pers.], and oats (*Avena sativa* L.). This compound also has been isolated from soils under allelopathic plants (Rice, 1984). Furmaric acid is a well-known microbial toxin produced by *Rhizopus* spp. in hull rot disease on almond (Mirocha et al., 1966). Asparagusic acid also appeared to be present in the inhibitory fractions. These compounds have been shown previously to be extremely active growth inhibitors (Takasugi et al., 1975; Yanagawa et al., 1972). Caffeic acid is reported to be fungistatic against *Helminthosporum carbonum* in potatoes and phytotoxic against many plant species and families (Rice, 1984). The fact that inhibitory activity of organic acids cannot be accounted for by pH also has been documented in the literature, specifically for malic and citric acid (Evanari, 1949). The standard organic acids were shown to be inhibitory in the cress bioassay system but never were sufficiently active to account for the activity of the crude extracts of asparagus root tissue. These compounds

might interact in an additive or synergistic manner with the isolated compounds. Young (1986) reported the isolation of several compounds from asparagus root exudates. These compounds were not present in any fractions obtained in our isolation procedures. Unfortunately, he did not test the activity of these compounds on asparagus or any other bioassay species. Furthermore, since his compounds were extracted from nonsterile sand, it is difficult to determine whether they were released from the asparagus root system or were microbial transformation products of the root exudates.

Methylenedioxycinnamic acid (MDCA) was isolated from fresh asparagus root tissue and was found to be more phytotoxic than any of the other compounds tested. Whether this compound is active as an allelochemical under natural conditions remains to be proven. Apparently, it was degraded or not extractable after the drying process used on the tissue. To our knowledge, this is the first report of the isolation and potential phytotoxicity of MDCA from asparagus or any other species.

Our experiments show that asparagus root tissues contain a number of inhibitory components important in the inhibition of seed germination and radicle elongation. These toxic components may be released from asparagus root tissue through exudation or degradation of the root tissue and could play a role in the alleged allelopathic activity of the asparagus plant. These data, as well as data presented elsewhere (Grogan and Kimble, 1959; Hartung and Stephens, 1983, 1984), suggest that the alelopathic potential of asparagus is probably not a result of any one chemical compound acting in isolation from other biotic factors associated with the asparagus agroecosystem but, instead, is caused by many chemical components that can act differently depending on soils, environmental conditions, and microbial populations. Further clarification of the chemicals released by asparagus root tissue and their relative biological activity in soils is needed to substantiate their role in causing asparagus decline.

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ALLELOCHEMICALS IN FOLIAGE OF UNFAVORED TREE HOSTS OF THE GYPSY MOTH, *Lymantria dispar* L.

1. Alkaloids and Other Components of *Liriodendron tulipifera* L. (Magnoliaceae), *Acer rubrum* L. (Aceraceae), and *Cornus florida* L. (Cornaceae)

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Abstract—Early theories on plant chemical defense against herbivory emphasized that apparent and unapparent plants were primarily defended by different types of compounds. More and more evidence suggests that both quantitative and qualitative defenses are found in apparent plants and that they can play a defensive role against herbivores. A survey of the literature on the gypsy moth suggests not only that there is a large variety of qualitative compounds, as well as the expected quantitative ones, but that unfavored hosts of the gypsy moth are associated with the presence of alkaloids. Foliage of three tree species, *Liriodendron tulipifera* L., *Acer rubrum* L., and *Cornus florida* L., was examined to confirm the presence of alkaloids and other major secondary metabolites. The known sesquiterpene lactone, lipiferolide, and the sugar derivative, liriodendritol, were components of *L. tulipifera* leaves, along with a bisphenylpropanoid previously found only in nutmeg. Alkaloid content [i.e., (−)-*N*-methylcrotsparine content] was low and leaves tested positive for HCN. Leaves of *A. rubrum* L. were examined for the presence of gramine, but none could be detected. No alkaloids were detected in *Cornus florida*.

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Key Words—*Lymantria dispar*, Lepidoptera, Lymantriidae, *Liriodendron tulipifera*, *Acer rubrum*, *Cornus florida*, gypsy moth, alkaloids, *N*-methylcrotsparine, 1-(3,4,5-trimethoxyphenyl)-2-(4-allyl-2,6-dimethoxy)propane, bisphenylpropanoid.

INTRODUCTION

The general theory of defensive chemistry of plants developed by Feeny (1976) and Rhoades and Cates (1976) suggests that "apparent plants" rely primarily on "quantitatively acting compounds" for their defense against herbivorous insects, rather than reliance on the "qualitatively acting compounds," which tend to be found in "unapparent plants." The role of alkaloids as defensive compounds is well known (Robinson, 1979; Wink, 1987). In experiments using primarily either unapparent plants or synthetic diets, alkaloids have been shown to be deterrent or toxic to a variety of insect herbivores (Bentley et al., 1984; Miller and Feeny, 1983; Zuniga and Corcuera, 1987; Wink, 1985, 1987). However, there is also evidence now that many apparent plants may gain protection from herbivores by producing qualitative compounds (Miller and Feeny, 1983; Bentley et al., 1984; Lindroth et al., 1986; Scriber et al., 1987; Mattson et al., 1988).

The influence of alkaloid distribution in plants on plant defense and in ecological interactions with other plant species has been discussed for arctic plants (Jung et al., 1979) and for tropical–temperate ecosystem comparisons (Levin, 1976; Levin and York, 1978). However, these reviews provided little or no insight into the relevance of alkaloid distribution to the defense of apparent and unapparent plants. In a survey of the literature, Barbosa and Krischik (1987) showed that many eastern North American tree genera contain many qualitative compounds that typically act as plant defenses. This survey also found that genera that are marginal and unfavored hosts of the gypsy moth (*Lymantria dispar* L.) contain alkaloids more often than favored hosts. If alkaloids are found to be important defenses in apparent plants, this would fail to support the qualitative–quantitative dichotomy suggested by the "plant apparency" theory.

The overall objective of our research is to determine if alkaloids in gypsy moth hosts do provide defense against this herbivore. The initial phase of this research, which is reported here, comprised an attempt to confirm the presence of alkaloids in the leaves of three eastern U.S. tree species that are not favored as hosts by the gypsy moth: tulip tree (*Liriodendron tulipifera* L.), flowering dogwood (*Cornus florida* L.), and red maple (*Acer rubrum* L.). Each of these species has been reported to contain alkaloids (Barbosa and Krischik, 1987). Leaves of the three species were to be used for the isolation, identification, and determination of the concentrations of specific alkaloids. Seasonal changes in

alkaloid content were to be measured so that subsequent experiments using artificial diets to determine the effects of alkaloids on the gypsy moth could be conducted at ecologically relevant concentrations.

Our primary interest was in the tulip tree, from which 40 different secondary metabolites, including many alkaloids, have been reported (Scriber et al., 1987; Ziyaev et al., 1987). One tulip tree alkaloid, glaucine, had been included in a group tested against the gypsy moth, but it was found to be only slightly toxic and did not affect larval consumption rates (Miller and Feeny, 1983). Earlier studies indicated that sesquiterpene lactones from the tulip tree showed antifeedant properties (Doskotch et al., 1981 and references therein). More recently five fractions of varying polarity, from an extraction of tulip tree leaves, were tested for feeding deterrency and their effect on the survival of *Papilio glaucus* larvae (Lindroth et al., 1986). Only one fraction was especially active, and this relatively nonpolar extract was thought to contain the sesquiterpene lactones. The fraction that was alkaloid-containing did not alter performance of the penultimate instar but did cause a moderate decline in neonate survival.

The study reported here extends the research noted above by identifying and quantifying specific alkaloids and other metabolites found in the tulip tree during the period of feeding by the gypsy moth. Since alkaloids have been reported to be present in the unfavored gypsy moth hosts, red maple and flowering dogwood, these species were also investigated for the presence of alkaloids.

METHODS AND MATERIALS

Foliage Collections. Leaves of *Liriodendron tulipifera*, *Acer rubrum*, and *Cornus florida* were collected by hand or using pole pruners, from a woodlot at the Beltsville Agricultural Research Center, USDA. Gypsy moth populations had been low in nearby areas in previous years, and none were observed during sampling for this study. Identified voucher specimens of each species were deposited at the Norton-Brown Herbarium, University of Maryland, College Park.

Two sampling procedures were used. For initial isolation of alkaloids and other compounds, bulk collections were made (e.g., 476 g of air-dried tulip tree leaves in June 1986, a similar bulk sample of dogwood leaves in June 1986, and 2 kg of fresh red maple leaves in September 1987) in which leaves from several trees were combined into single, large samples. To detect chronological trends and tree-to-tree variability in the concentration of these compounds, a more detailed sampling scheme was used. In 1987, trees were sampled weekly, starting on March 29 and extending through the larval development time of the

gypsy moth. Two 15-g foliage samples were taken from each of four trees of each species each week. Leaves of each tree were randomly sampled in such a way that the same area of the tree was never sampled twice. A ring marked with 20 equally spaced compass directions was placed around the base of the trunk. Using a random numbers table, two different compass directions were picked each week. Each 15-g foliage sample was taken from an area of the crown intersecting a plane vertical to one of the chosen compass directions. Each sample was returned to the laboratory in a plastic zip-lock bag and divided into three 5-g subsamples. Each subsample was either air dried for about three days, placed in methanol, or freeze dried.

Isolation Procedures (Liriodendron tulipifera). Two separate methods were employed, one for nonalkaloidal material and the other for alkaloids. For nonalkaloidal material, dried and ground leaves were extracted (Soxhlet) with hexane and then chloroform (24 hr each) and the extracts condensed to small, gummy residues in vacuo (in a rotary evaporator). These residues were processed via chromatographic procedures (see Results).

To check for the presence of HCN, we prepared Feigl-Anger test strips (D. Seigler, personal communication; Feigl and Anger, 1966). A 1% (w/v) solution of 4,4-tetramethyldiaminodiphenylmethane in chloroform and a 1% (w/v) solution of copper ethyl acetoacetate in chloroform were prepared. Equal volumes of the two solutions were mixed, and strips of Whatman No. 3MM filter paper were soaked for about 2 min in the mixture and dried. We tested the following materials for HCN: six leaves of tulip tree, two leaves of *Catalpa speciosa* Warder, potassium cyanide, and deionized water. Each leaf was ground individually in a mortar and pestle and placed in a 5.3-ml (3-dram) glass vial with enough deionized water to fill the vial to one third of its volume. Each vial was corked, and a test strip was hung in the vial. After 24 hr, we graded the color of each test strip using the Methuen Handbook of Colour (Kornerup et al., 1984).

For the isolation of alkaloids, sufficient 10% aq. $NaHCO_3$ was added to the plant material to wet it and then a 1:1 1-butanol–toluene solution was added, approximately 10 ml/g of plant. This was stirred well and allowed to stand for 24 hr for large extractions, or for 5 hr with constant stirring for small (5-g) plant samples. The mixture was filtered and the alkaloids extracted into acid. Either 1 M H_2SO_4, 1 M HCl, or pH 4 tartaric acid solution was used. The acidic solution was extracted with $CHCl_3$ to remove nonalkaloidal materials and then made basic to pH 9 with NH_4OH or NaOH pellets. This solution was then extracted four times with equal volumes of $CHCl_3$, the $CHCl_3$ solutions were combined, dried over anhydrous Na_2SO_4, and the alkaloid residue weighed. Thin-layer chromatography (silica gel, chloroform–methanol 7:3 elution, and iodoplatinate visualization) was used to analyze the mixed alkaloid residue. Although extraction yields of alkaloids were low and highly variable, this var-

iability did not appear to be associated with the method of leaf preservation, i.e., immersion in methanol, freeze drying, or air drying.

Isolation Studies (Acer rubrum *and* Cornus florida). The 2-kg sample of *Acer rubrum* (collected in September 1987) was percolated with MeOH–EtOH and the dried extract partitioned repeatedly between 0.5 M HCl and $CHCl_3$. The acid layer was altered to a pH of 9 with 5 M NaOH (in ice) and extracted with $CHCl_3$ ($\times 3$). The $CHCl_3$ extract was dried over anhydrous sodium sulfate and evaporated to yield a crude alkaloid fraction (4.4 mg), which showed one low R_f spot by TLC (Silica gel, $CHCl_3$–MeOH 7:3, iodoplatinic acid spray). Analysis of the individual 5-g *Acer rubrum* subsamples, collected from May to June 1987 was undertaken using the same procedures.

A similar large-scale extraction and isolation of an alkaloid residue was attempted on air-dried leaves of *Cornus florida*.

RESULTS

L. tulipifera *Non-alkaloidal Metabolites*. The hexane extract residue (10 g) from the 476-g bulk sample of dry leaves was chromatographed (flash column, silica gel, hexane followed by increasing amounts of ethyl acetate), and various fractions were examined by TLC and [^{1}H]NMR spectroscopy. Most fractions contained only alkane or lipid-like material, and only the most polar eluting fractions were combined to yield 74 mg of residue. Preparative layer chromatography (silica gel, ethyl ether–chloroform–hexane 3:1:1) was used to isolate a small amount of lipiferolide, **1** (Figure 1), a sesquiterpene lactone (more easily isolated from the chloroform extract; see below) and 40 mg of a yellow oil whose molecular formula was determined to be $C_{23}H_{30}O_6$ by high-resolution mass spectrometry. The [^{1}H]NMR, IR, and UV spectra, were the same as those reported in the literature for the bisphenylpropanoid, **2** (Figure 1), namely, 1-(3,4,5-trimethoxyphenyl)-2-(4-allyl-2,6-dimethoxy)propane (Isogai et al., 1973; Forrest et al., 1974). The [^{13}C]NMR spectrum, not previously reported, was also consistent with structure **2** (Figure 1): 153.9 and 153.1 ppm (2s; C3, C5, C2′, C6′), 137.3 (d; Cβ'), 135.4 and 134.8 (2s; C1, C1′, C4, C4′), 115.7 (t; Cγ'), 107.5 and 106.4 (2s; C2, C3′, C5′, C6), 79.6 (d; Cβ), 60.7 (q; C4 OMe), 56.3 and 56.2 (2q; C3, C5, C2′, and C6′ OMe's), 43.7 and 40.4 (2t; α and α'), 19.7 (q; Cγ).

When the chloroform extract of the same bulk sample had been evaporated to a small volume, 1.3 g of crystalline material precipitated. This was identified as liriodendritol, i.e., 1,4-di-*O*-methylinositol, **3** (Figure 1), by comparison of mp, [^{13}C]NMR and mass spectra with literature values (Angyal and Bender, 1961; Breitmaier and Voelter, 1978). The mother liquor was evaporated to dryness (34.2 g) and the residue partitioned between aqueous methanol and hexane.

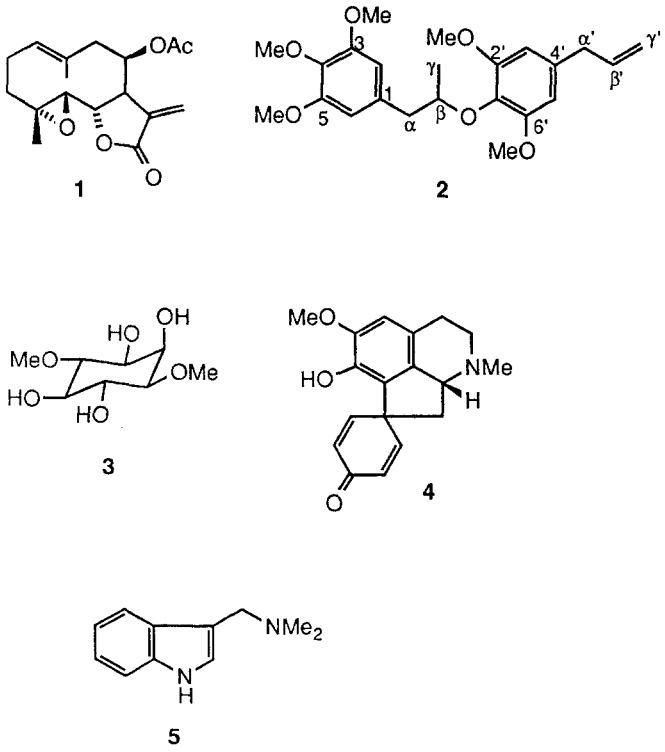

FIG. 1. Components of *Liriodendron tulipifera*: **1** (lipiferolide), **2** [1-(3,4,5-trimethoxyphenyl)-2-(4-allyl-2,6-dimethoxy)propane], **3** (liriodendritol), **4** (*N*-methylcrotsparine); and *Acer rubrum*: **5** (gramine).

The methanol was removed from aqueous methanol layer, and the aqueous solution remaining was extracted with ethyl acetate and then 1-butanol. An additional 491 mg of liriodendritol was recovered from the water and butanol layers. The ethyl acetate layer was evaporated to dryness to leave 2.9 g of residue, which was chromatographed (silica gel, chloroform with increasing amounts of methanol) to yield 158 mg of yellow gum (10% methanol in chloroform fractions) identified as lipiferolide, **1** (Figure 1), by comparison of [^{1}H]- and [^{13}C]NMR spectral and optical rotation with those previously reported (Doskotch et al., 1975) and with a standard sample. Additional amounts of the bisphenylpropanoid, **2** (Figure 1), were detected in early fractions from the chromatography. A recent report has described the isolation of this compound from seeds of *L. tulipifera* (Muhammad and Hufford, 1989).

L. tulipifera *Alkaloids.* From the same bulk sample of dried June leaves,

we isolated the proaporphine alkaloid (−)-*N*-methylcrotsparine, **4** (Figure 1), as the major alkaloid. Identification was by optical rotation, mass spectrum, and [^{1}H]- and [^{13}C]NMR spectra in comparison to values in the literature. Smaller amounts of other alkaloids could be detected by heavy spotting of TLC samples but were not further investigated. The total alkaloid yield varied from 0.01 to 0.08% of the dry weight, depending upon the acid used in the isolation procedure. Use of 0.5 M HCl and rapid extraction seemed to give the best yield, which also occurred with small leaf samples. When large samples were extracted, the procedure was slower, and alkaloids remained in acid for a longer time. Proaporphine alkaloids are acid-sensitive and losses may occur depending upon the acid strength used in the isolation procedure. This might account for our higher yield than that of 0.003% reported by Lindroth et al. (1986), where a somewhat different extraction scheme was used. On the other hand, our results were much lower than the 0.1–0.3% total alkaloids reported by Ziyaev et al. (1975) from leaves at various growth stages. The lability and low concentration of **4** (Figure 1) did not permit our gravimetric analysis method to resolve differences among methods of leaf preservation, samples taken over time, or among trees of a species.

Semiquantitative analyses of the 5-g subsamples from the 1987 sampling scheme (using TLC spot size in comparison to a standard) indicated that (−)-*N*-methylcrotsparine was present during the entire sampling period. This method showed little difference in concentration between air- and freeze-dried samples.

Finally, *L. tulipifera* foliage was found to contain HCN. Tulip tree leaves rapidly caused six test strips to change color, ranging from blue to deep purple [see Methuen Handbook of Colour (Kornerup et al., 1984), p. 18: C8, D8, and E8; and p. 23: C7 and D8], indicating the presence of HCN. The positive control, potassium cyanide, turned deep violet (Kornerup et al., 1984, p. 18: E8). The negative controls, water, and catalpa leaves failed to produce a change in test strips.

Acer rubrum. From 2 kg of fresh leaf material, collected in September 1987, we isolated 4.4 mg (less than 0.001% based on dry weight) of gramine, **5** (Figure 1). This is significantly less than the 0.03% reported from fruits of *A. rubrum* (Pachter, 1959) and far less than the 100–200 mg/kg wet weight reported for barley seedlings (Zuniga et al., 1985). No gramine could be detected from any of the weekly leaf subsamples (i.e., the 5-g samples) collected from May to June of 1987, even after TLC application of the entire alkaloid extract. This failure can be attributed to the low concentrations present, which were well below the minimum detectable limits of the TLC technique used.

Cornus florida. Although Willaman and Schubert (1961) reported the presence of an unknown alkaloid in leaf and stem tissues, no alkaloids were detected in our large-scale leaf extracts.

DISCUSSION

Liriodendron tulipifera. Although over 40 secondary metabolites have been reported from this tree (Scriber et al., 1987; Ziyaev, 1987), we were able to identify one more, a bisphenylpropanoid, **2** (Figure 1), not previously reported from *L. tulipifera*. As far as we are aware, it has been found previously only in nutmeg (or mace), i.e., in the seeds of *Myristica fragrans* Houtt (Isogai et al., 1973; Forrest et al., 1974). Nutmeg was reported to inhibit growth of silkworm larvae, but it contains many other secondary metabolites in addition to numerous bisphenylpropanoids (Isogai et al., 1973; Hattori et al., 1987; Nakamura et al., 1988).

The compound that we found in large quantity, liriodendritol, was recently shown to be absent in young leaves and synthesized only in fully expanded leaves (Dittrich and Schilling, 1988). Tulip trees break buds early in the eastern United States, and leaves are fully expanded by June. The majority of feeding by gypsy moth larvae is accomplished by the ultimate and penultimate instars, which usually occur in June.

Although we did not quantify HCN content, Hegnauer (1958) reported a concentration of 225–248 mg/kg of "June" leaves and Mirande (1913), 490 mg/kg of "May" leaves. The high cyanide content, at a time when gypsy moth larvae are present, could act as an antifeedant or antibiotic, but this has not been tested (Doskotch et al., 1981; Scriber et al., 1987).

The literature contains many reports on alkaloids of *L. tulipifera*, but nearly all deal with analysis of plant parts other than leaves, in particular of outer wood and heart wood. The main exception is that of Ziyaev et al. (1975), who reported leaf alkaloid content of 27- to 30-year-old *L. tulipifera* at the Botanical Garden of the Uzbek SSR Academy of Science (Tashkent, U.S.S.R.), at the beginning of flowering (0.32%), the end of flowering (0.30%), time of fruiting (0.23%), and yellowing (0.11%). Only the first two sampling periods correspond to the times of our leaf collections, and individual alkaloid isolation was not reported by Ziyaev for these early growth periods, only for the latter two cases. At the time of fruiting, only aporphine alkaloids were identified, whereas yellow leaves contained aporphines along with the *N*-methylcrotsparine reported in this study. The alkaloid glaucine, which was reported by William and Liu (1970), was not reported by Ziyaev et al. (1975), nor did we find it in our study. It is probably only a wood alkaloid and hence of no importance to gypsy moth larval feeding. In one of the few other reports on leaf alkaloids of *L. tulipifera*, Tomita and Furukawa (1962) suggested that glaucine was present in trace amounts, but this was based only on a paper chromatographic test, and the spot was not identified further. Using March leaves, Tomita and Furukawa (1962) also noted a small amount of an unknown phenolic base that was too unstable to isolate.

N-Methylcrotsparine is undoubtedly the biosynthetic precursor of most of

the leaf aporphines that occur at later growth stages, and our finding of this alkaloid as the major component in early season leaves is therefore expected. *N*-Methylcrotsparine has been reported to have hypotensive activity (Ishiwatari et al., 1974), and its enantiomer, glaziovine, is reported to have potential as a tranquilizer (Casagrande and Canonica, 1974a,b). Potent antibacterial activity against both gram-positive and gram-negative bacteria was recently reported with an extract of *L. tulipifera* leaves, but the active ingredient was not isolated (Bae and Byun, 1987).

Acer rubrum *and* Cornus florida. Although gramine has been implicated as a deterrent to insect feeding for some plants (Zuniga et al., 1985; Zuniga and Corcuera, 1987), the extremely low level of gramine found in leaves of *A. rubrum* may make it an unlikely deterrent to gypsy moth feeding.

C. florida was reported to contain alkaloids in a compilation of literature reports for 3671 species (Willaman and Schubert, 1961). The positive result was part of a survey of over 900 species for the presence of alkaloids (Wall et al., 1959). Individual data from large surveys of this sort nearly always need confirmation before their results can be properly assessed. Alkaloid screening tests that employ NH_3 in the procedure can create alkaloids from iridoids artifactually, and this could be the source of false alkaloid tests reported for *C. florida*.

Cornus florida has been reported (Hostettmann et al., 1978) to contain molluscicidal saponins. Since the association between the presence of saponins and the unfavorableness of host plants to the gypsy moth approached statistical significance (Barbosa and Krischik, 1987), it is possible that it is the saponin content of *C. florida* that is deterrent. One also should note, however, that the allelochemical category "iridoids" was not included in the Barbosa and Krischik (1987) survey, and the unfavored families Bignoniaceae, Caprifoliaceae, Cornaceae, Ericaceae and Oleaceae are all iridoid-containing. Iridoid glucosides have been found in *C. florida* (Jensen et al., 1975). In addition, in a study similar to that of Barbosa and Krischik (1987), Hanson and Miller (unpublished data) noted that iridoids as well as alkaloids correlated positively with unfavorableness of host plants of the gypsy moth in the western United States.

These results suggest, as a first approximation, that using data on secondary metabolites (such as alkaloids) at the generic level to suggest their presence in any species of the genus (Barbosa and Krischik, 1987) may have some validity, but it is subject to a high margin of error. In general, although alkaloids have been reported to occur in the three species examined, alkaloids were either absent in the leaves of red maple and flowering dogwood or occurred in very low concentrations. Only one major alkaloid was found in early season tulip tree foliage, and the previously reported occurrence of the antifeedant lipiferolide was confirmed. In addition, a new foliage metabolite, the bisphenylpropanoid, **2** (Figure 1), was identified. Future feeding experiments incorporating

some of these potentially important allelochemicals into diets will elucidate their biological activity for the gypsy moth and help determine the relative importance of qualitative defenses in apparent plants.

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ALLELOCHEMICALS IN FOLIAGE OF UNFAVORED TREE HOSTS OF THE GYPSY MOTH, *Lymantria dispar* L.
2. Seasonal Variation of Saponins in *Ilex opaca* and Identification of Saponin Aglycones

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Abstract—A greater variety of qualitative chemical defenses has been reported in eastern forest trees than might be expected from current interpretation of the plant apparency theory. For the gypsy moth there is an association between the occurrence of alkaloids and unfavorability of certain tree species, as well as the presence of saponins. The latter association, however, is not statistically significant. Species in the genus *Ilex* have been reported to contain both alkaloids and saponins (Barbosa and Krischick, 1987). In this study, determinations were made of the occurrence of alkaloids and saponins in *I. opaca* and their changes in concentration over time. No alkaloids were detected. Saponins were isolated, and the aglycone siaresinolic acid was identified. Saponin concentration changes seasonally, being highest in early May and lowest in early June leaves.

Key Words—*Ilex opaca*, Aquifoliaceae, *Lymantria dispar*, gypsy moth, Lepidoptera, Lymantriidae, saponins, siaresinolic acid, qualitative plant defenses.

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INTRODUCTION

Barbosa and Krischik (1987) pointed out that the role of qualitative, i.e., mobile (Coley et al., 1985) defenses in apparent (predictable, slow-growing, long-lived) plant species rarely has been considered or evaluated experimentally. A survey of the chemistry of nonfavored temperate tree hosts of the gypsy moth *Lymantria dispar* L. (Barbosa and Krischik, 1987) uncovered a surprising number of reports of qualitative allelochemicals (cyanogenic glucosides, saponins, coumarins, terpenoids, alkaloids, etc.) along with the expected quantitative defenses such as tannins. Although tannins may be important as defenses among hosts of the gypsy moth, Barbosa and Krischik (1987) found that preference of gypsy moth larvae for plant genera or species was negatively associated with the presence of alkaloids in these plant species. This suggested that the reason for the unfavorableness of many plant species to the gypsy moth may be the content of qualitative compounds in the foliage of these species.

One common but unfavored tree species is *Ilex opaca* Ait., American holly. Some species in the genus *Ilex* have been reported to contain both alkaloids and saponins. Saponins also were found to be associated with unfavorability of plant species to the gypsy moth (Barbosa and Krischik, 1987), although the significance levels was 0.09. Saponins have been isolated from ethanol extracts of *I. opaca* leaves (West et al., 1977). One mixture, after hydrolysis, yielded 0.072% of the triterpene oleanolic acid (West et al., 1977). Thin-layer chromatography after the acid hydrolysis of another crude saponin preparation indicated that this was a glucosidic mixture of three additional unknown aglycones.

The American holly, *I. opaca* is of particular interest in the evaluation of the relative importance of qualitative and quantitative defenses because it is a relatively apparent, woody species, known to have qualitative defenses (saponins and phenolics) at early growth stages (Potter and Kimmerer, 1986), and the genus *Ilex* is also reported to contain quantitative defenses (e.g., tannins) (Barbosa and Krischik, 1987). Some species of *Ilex*, such as *I. paraguensis*, contain caffeinelike alkaloids (Hegnauer, 1964). The ecological importance of the nutritional, chemical, and physical aspects of *I. opaca* in its defense against the specialist leafminer, *Phytomyza iliciola*, and the fall webworm, *Hyphantria cunea*, has been discussed by Potter and Kimmerer (1986, 1988).

Our goal in this study was to confirm the presence of saponins and alkaloids in *I. opaca* and assess concentration changes over time in order to establish appropriate, ecologically relevant levels for future artificial diet feeding studies using the gypsy moth, a generalist. Because little detailed chemical work has been reported on *I. opaca*, a second goal was to identify some of the additional aglycones or saponins.

METHODS AND MATERIALS

Ilex opaca Ait. (Aquifoliaceae) was collected at the USDA, Beltsville Agricultural Research Center. Foliage sampling was done in two stages. In the summer of 1986, large, unreplicated samples were taken to provide bulk foliage batches for initial isolation studies. To obtain more precise information on seasonal changes in concentration of allelochemicals, a systematic sampling scheme was instituted in 1987 (see below). A voucher specimen was deposited at the Norton-Brown Herbarium, University of Maryland, College Park.

Isolation of Saponins. Ground, air-dried *I. opaca* leaves (636 g), collected in the summer of 1986, were extracted (Soxhlet) with methanol for 72 hr. The solvent was removed in vacuo and the residue suspended in water and extracted successively with ether, ethyl acetate, and 1-butanol. Evaporation of the butanol left a saponin residue. This crude saponin extract was subjected to TLC (silica gel; $CHCl_3$–MeOH–H_2O 10:5:1) with *p*-anisaldehyde–H_2SO_4 visualization. Of the residue, 23.5 g was heated at reflux for 5 hr in 1:1 EtOH–1 M HCl. To this was added 40 ml of water. The solution was condensed to half its volume, and the resulting brown precipitate was filtered, washed with water, and dried to yield 5.7 g of a crude aglycone brown powder, which was then purified by flash column chromatography (silica gel, $CHCl_3$ with increasing amounts of MeOH).

Determination of Saponin Concentration in Leaves. Semiquantitative analyses were carried out on *Ilex opaca* leaf samples collected once weekly from May 7 to June 24, 1987. Sampling and preservation methods for *I. opaca* leaves were identical to those reported by Barbosa et al. (1990) for red maple, tulip tree, and dogwood, except for the following modifications: Instead of taking two 15-g leaf samples from each of four trees each week, we, at first, sampled four groups of trees with five trees in each group and took two 3-g samples from each tree. The total amount of leaf material sampled from each of the four groups of *I. opaca* trees was therefore the same as that taken from each of the four trees of each of the other three species. Lighter sampling from each *I. opaca* tree was necessary because new leaves on *I. opaca* expand at a much slower rate than new leaves of the other three species. Each 3-g sample was subdivided into three 1-g subsamples, which were preserved as described in Barbosa et al. (1990). After leaves were fully expanded on *I. opaca* (i.e., beginning May 20), we randomly selected one tree from each of the four groups and increased the amount of foliage sampled to 30 g per tree per week.

Because of time limitations, only the air-dried subsamples were analyzed. Each week, two of these samples from each tree were weighed and extracted by percolation with 40 ml of 0.9% w/v NaCl solution. For each extract, a

dilution series was constructed in isotonic pH 7 buffer, to give a range of concentrations from 100 to 0.1% of the original. To each dilution (2 ml) was added 0.200 ml of a red blood cell suspension, which was prepared as follows: Fifteen milliliters of fresh human blood was centrifuged. The red blood cells were collected, diluted with 25 ml of saline, and filtered through glass wool. The filtrate was then centrifuged, and the red blood cells were collected. The last step was repeated until the supernatant wash was clear after centrifugation. The washed red blood cells were made up to 4 ml with isotonic buffer, and 2 ml of this suspension was diluted to 100 ml with the buffer. This was the stock suspension, from which 0.200 ml was used as described above.

The samples were allowed to stand for 3 hr at 25°C, after which time the extent of hemolysis was assessed by visual comparison to a standard digitonin solution. These results then were converted into real saponin concentrations by comparison of the hemolytic activity of a crude *I. opaca* saponin extract with that of digitonin. The hemolytic response of 0.033 mg of crude *I. opaca* saponins gave the same hemolytic response as 0.002 mg of digitonin.

Isolation of Alkaloids. Sufficient 10% aq. $NaHCO_3$ was added to the leaf material to wet it, and then a 1:1 1-butanol–toluene solution was added at approximately 10 ml/g of leaf material. This was stirred well and allowed to stand for 24 hr for large samples, or for 5 hr with constant stirring for small (5-g) plant samples. The mixture was filtered and the alkaloids were extracted into 1 M H_2SO_4. The acidic solution was extracted with $CHCl_3$ to remove nonalkaloidal materials and then was made basic to pH 9 with aq. NH_4OH. This solution then was extracted four times with equal volumes of $CHCl_3$. The $CHCl_3$ solutions were combined, dried over anhydrous Na_2SO_4, and the residue tested for alkaloids by thin-layer chromatography (silica gel, chloroform–methanol 7:3 elution, and iodoplatinate visualization).

RESULTS

Flash column chromatography of the saponin aglycone mixture yielded two white powder fractions during the 10% MeOH in $CHCl_3$ elution. The first fraction was dissolved in hot DMSO–EtOH, and the EtOH was removed in vacuo. The 100-mg precipitate was identified as 3β, 19α-dihydroxyolean-12-en-28-oic acid (siaresolinic acid), **1** (Figure 1), by [^{1}H]- and [^{13}C]NMR spectra, mass spectrum, and conversion to methyl siaresolinate, which was compared (mp, optical rotation, TLC and [^{1}H]NMR spectrum) to a standard sample (Inada et al., 1987).

The other white powder fraction was treated similarly to yield 150 mg of precipitate, which was methylated directly with diazomethane. The [^{13}C]NMR spectrum indicated that it consisted of a mixture of two closely related substances, which were also closely related to **1** (Figure 1). The mass spectrum

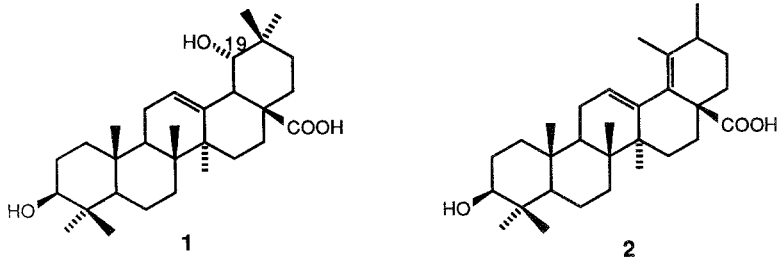

FIG. 1. Saponin aglycones from *Ilex opaca*: **1** (siaresinolic acid), and **2** (a dehydro urs-12-en-28-oic acid).

gave M^+ 468, which is consistent with a $C_{31}H_{48}O_3$ formulation, or the same molecular formula as **1** (Figure 1), less H_2O. The [^{1}H]NMR spectrum showed a singlet methyl resonance at 1.73 ppm, which would be expected for an alkenyl methyl. Such a resonance is absent in the spectrum of **1** (Figure 1). This is as expected for the methyl ester of a dehydro urs-12-en-28-oic acid such as **2** (Figure 1) (Nakanishi et al., 1982), derived from a saponin aglycone similar to **1** (Figure 1) by elimination of water from the 19α-OH. A double bond isomer of **2** (Figure 1) would probably be the second component of the mixture. Standard ilexoside A (Inada et al., 1987), the xylopyranoside of **1** (Figure 1), ran near the solvent front in our TLC system, whereas spots we observed in the crude saponin extract were all of lower R_f value. Most of the *I. opaca* saponins are thus likely to be di- or trisaccharides.

The saponin concentration of *I. opaca* leaves decreased from a maximum of about 125 mg/g in early May to generally less than 10 mg/g by early June, based upon the human blood cell hemolysis bioassay in comparison with digitonin (Figure 2).

Tests for the presence of alkaloids in *I. opaca* leaves were negative. We used commercial *I. paraquensis* (South American maté) as a test of our isolation scheme and found high levels of alkaloids. Thus, our procedures were valid for isolation of purine-type alkaloids.

DISCUSSION

Although the role of saponins in gypsy moth survival and development is unknown, saponins are known to be important in other plant–insect interactions (Sutherland et al., 1975; Applebaum and Birk, 1979; Kimmerer and Potter, 1986; Ishaaya, 1986; Jain, 1987). Because they occur in over 500 species in 80 families (Applebaum and Birk, 1979), their importance may become more evident as research on this class of allelochemicals continues.

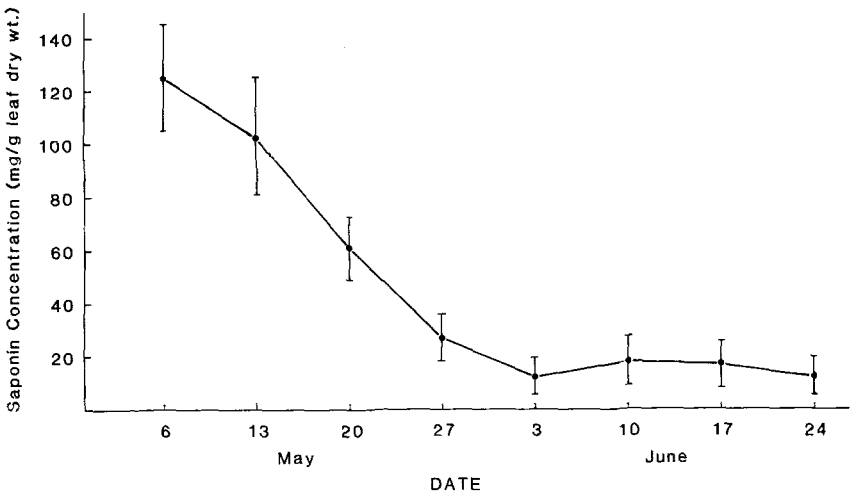

FIG. 2. Change in saponin concentration of *Ilex opaca* leaves from May through June.

Seasonal changes in plants that affect herbivore fitness and survival have been documented in a variety of species (Barbosa and Wagner, 1989). These include changes in the presence and concentration of various compounds that serve as nutrients for herbivores or as repellent (or toxic) allelochemicals. These and other seasonal changes can determine the vulnerability of plants to herbivore feeding and the portion of the season in which injury, if any, is likely to occur. Thus, the changes in saponin concentration may, in part, determine the vulnerability of holly. The seasonal change in saponin concentration that we observed during early leaf development was virtually identical to that determined by Potter and Kimmerer (1986) for *I. opaca* in Kentucky. Mean values from five trees were about 125 mg/g dry weight in early May and dropped to about 10 mg/g in early June. This pattern may reflect a chemical plant defense that protects new, relatively soft foliage from injury by early- to mid-season herbivores such as the gypsy moth. Young leaves of *Ilex opaca* are unsuitable to the southern red mite and cause a significant reduction in pupal weight and an increase in mortality of the fall webworm (D. Potter, personal communication). As saponin concentration drops, other potential defenses, such as leaf toughness and low water and nitrogen content (Potter and Kimmerer, 1986), may provide protection against herbivory and other injury. Other nutritional deficiencies and/or chemical defenses could also be present. Willems (1988) reported that *Ilex aquifolium* contained a cyanogenic glucoside.

The complex chemical nature of the saponin mixture that we found is similar to that reported by West et al. (1977), except that we did not find oleanolic

acid as a major aglycone; instead, we found **1** (Figure 1) and the dienic ursolic acid derivative related to **2** (Figure 1). Siaresolinic acid was reported for the first time recently as a major aglycone from the hydrolysis of a saponin mixture from *I. chinensis* (Inada et al., 1987). The dienic aglycone mixture we obtained was quite similar to that produced as an artifact from acid rearrangement of pomolic acid (Inada et al., 1987). Thus, pomolic acid is likely to be one of the *I. opaca* aglycones.

The results of this study and those of Barbosa et al. (1990) will be used to design future experiments in which saponins and alkaloids, at ecologically relevant concentrations, will be incorporated into synthetic diet to determine their influence on the gypsy moth.

Acknowledgments—We thank Professor Akira Inada for standard samples of methyl siaresinolate and ilexoside A. D. Laughlin and W. Wooge provided technical assistance with foliage sampling. Plant specimen identification was provided by G. Eisenbeiss of the National Arboretum, U.S. Department of Agriculture, Washington, D.C. Figure 2 was drawn by Ms. A. Hsu. Scientific contribution No. 8065, article No. A-5017 of the Maryland Agricultural Experiment Station, Department of Entomology. This research is supported by NSF grant BSR-8604303.

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Letter to the Editor

BACTERIAL DEGRADATION OF JUGLONE
Evidence Against Allelopathy?

Recently, Schmidt (1988) isolated from the soil under *Juglans nigra* L. a bacterium capable of degrading juglone, the allelotoxin reputed to inhibit neighboring vegetation (Davis, 1928; Rietveld, 1983; Rietveld et al., 1983). Schmidt's report is noteworthy because it supports the previously hypothesized degradation of juglone by microorganisms (Fisher, 1978; Rietveld, 1983; Rietveld et al., 1983). However, Schmidt's (1988, p. 1561) conclusion that "Rapid degradation of juglone and other suspected allelochemicals by soil bacteria make it unlikely that these compounds are important mediators of plant–plant interactions under natural conditions" appears to us to be premature. In fact, the author might have argued equally convincingly that such specialized strains of soil bacteria confirm the frequent, if not continuous, presence of putative phytotoxins in the soil, and therein lend support to the allelopathic hypothesis. Neither contention is free of ambiguity.

The phytotoxicity of allelochemicals in soil solution will depend on input and output rates as well as the effective concentration (Winter, 1961; Blum and Shafer, 1988). Schmidt's *Pseudomonas* J1 provides one output sink for juglone. Juglone may be removed from the soil in numerous other ways, including, as Schmidt notes, soil physical and chemical processes. However, just as plants compete with microorganisms for nutrients (Pastor et al., 1984), the roots of plants inhibited by phytotoxins are potential competitors with microorganisms for the available phytotoxins (Winter, 1961; Hoffman and Lavy, 1978; Weidenhamer et al., 1987, 1989). The outcome of this competition may determine whether or not allelopathic effects are manifested. Therefore, bacterial affinities for juglone must be evaluated relative to target plant affinities and soil abiotic processes.

Output rates themselves must be weighed against input rates of juglone into the soil solution (Winter, 1961). Influx may be a function of the amount of plant biomass (Rietveld et al., 1983), root exudation, litter decay, and precipitation throughfall, although in the case of juglone little is known in this regard.

The actual available concentration of juglone will reflect the various input

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and output rates. Toxicity is likely to be a function of both concentration and flux, where the former measures static availability and the latter measures dynamic or potential availability. Static availability is the existing concentration in soil solution, while dynamic availability is the renewal rate (input or production measured under controlled output). In order to determine the dynamic availability of compounds in relation to processes affecting them (Vitousek et al., 1982), chemical ecologists may resort to controlled incubation techniques, analogous to those employed to estimate available soil nutrients (Keeney, 1980; Powers, 1980). Until such studies are carried out, it would be premature to disavow the involvement of juglone in allelopathic interactions.

In fact, evidence already exists contrary to Schmidt's conclusion that juglone concentrations in soil are maintained below phytotoxic levels by *Pseudomonas* J1 and other bacteria. Ponder and Tadros (1985) found concentrations of juglone under black walnuts to be 3.6–4.0 μg/g in surface soils—potentially sufficient to produce a soil solution of 10^{-4} M, a level known to cause toxic effects in the laboratory (Funk et al., 1979; Rietveld, 1983) and well above those mineralized by *Pseudomonas* J1. In addition, recent experiments with tomatoes grown in unsterilized black walnut soil demonstrated density-dependent effects on growth, characteristic of the presence of toxic substances in soil (Weidenhamer et al., 1989). If microbes are "literally waiting to consume compounds such as juglone" (Schmidt, p. 1569), they appear not to be as effective at scavenging juglone in the field soils as Schmidt's lab data would suggest. Furthermore, Schmidt isolated *Pseudomonas* J1 from only three of five soil samples. These microorganisms may be distributed patchily or respond facultatively to juglone enrichment only under certain conditions. For example, ferulic acid, when applied experimentally to soils, was readily degraded by microorganisms under nutrient-rich conditions but accumulated temporarily under nutrient limitation (Blum and Shafer, 1989).

Finally, we note that microorganisms can toxify as well as detoxify secondary compounds. In soil, transformation of allelochemicals seems to be common, but the outcome is not necessarily detoxification (Einhellig, 1986; Liebl and Worsham, 1983; Kaminsky 1981).

Much remains to be learned about the fate of plant allelochemicals in the environment. We heartily endorse Schmidt's call for tests of allelochemical effects in unsterilized soils under natural field conditions, especially combined with the more traditional studies in sterile soil or artificial media, and his admonition to question allelopathic claims based only on the latter. However, let us not discard the allelopathic hypothesis as perfunctorily as it may have been accepted (Harper, 1975, 1977; Williamson, 1990).

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ORIGIN OF KAIROMONES IN THE LEEK MOTH (*Acrolepiopsis assectella*, Lep.) FRASS
Possible Pathway from Methylthio to Propylthio Compounds

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Abstract—Feeding leek moths on an artificial diet has shown that dimethyl and dipropyl disulfides and methyl-propyl disulfide found in frass arise from sulfur compounds specific to *Allium*. The addition of either propyl or methyl disulfide or their precursors to the diet leads to appearance of the three disulfides in the frass. This implies the transformation of the *S*-propyl moiety to *S*-methyl and vice versa by an as yet unknown mechanism.

Key Words—*Diadromus pulchellus*, Hymenoptera, Ichneumonidae, *Acrolepiopsis assectella*, Lepidoptera, Acrolepiidae, leek, frass, methyl disulfide, propyl disulfide, kairomones, host plant.

INTRODUCTION

Frass volatiles of the leek moth *Acrolepiopsis assectella* Zell. (Lepidoptera: Acrolepiidae) contains dimethyl disulfide (Me_2S_2) and dipropyl disulfide (Pr_2S_2), as well as the mixed methyl-propyl disulfide ($MePrS_2$). These substances function as kairomones for *Diadromus pulchellus* Wesm. (Hymenoptera: Ichneumonidae), a solitary endoparasitoide of young leek moth nymphs (Auger et al., 1989a; Thibout et al., 1988).

Propyl disulfide is the major sulfur compound in leek volatiles, while methyl and methyl-propyl disulfides are far less abundant (Schreyen et al., 1976). It is highly probable that these disulfides contained in the frass arise, as they do in the leek, from *S*-propyl cysteine sulfoxide (PCSO) and *S*-methyl cysteine sulfoxide (MCSO) (Auger, 1987; Auger et al., 1989b). In the course of feeding, larva ingest these precursors or substances arising from them, such

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as thiosulfinates and disulfides. The former could form when cells are disrupted by chewing larvae, and these substances can persist in the atmosphere for a certain period of time. In fact the stability of thiosulfinates is not known in vapor phase, but they disproportionate spontaneously in pure liquid state (Auger, 1987). Disulfides arise from either the transformation of thiosulfinates (Auger et al., 1989b) or directly from the breakdown of precursors by microorganisms (Murakami, 1960).

The relative abundance of methyl disulfides in the feces could be due to a difference in digestive metabolism of ingested sulfur-containing substances or to an origin of these disulfides different from that invoked above. In this case, they would arise from the transformation of precursors other than *Allium*-specific *S*-alkyl cysteine sulfoxides. Thus, the breakdown of numerous sulfur substances yields dimethyl sulfide and sometimes dimethyl disulfide (Murakami, 1960).

The aim of our work was to define the vegetal origin and the nature of the volatiles' precursors by analyzing the frass odors of leek moths fed an artificial diet lacking *Allium* and supplemented with one of the following compounds: leek powder, Pr_2S_2, Me_2S_2, PCSO, or MCSO.

METHODS AND MATERIALS

Instrumentation. Analytical gas chromatography (GC) was carried out using a Varian 3300 chromatograph equipped with on-column injection and two detectors, FID and FPD in the sulfur mode. The capillary columns used were SGE fused silica 25 m long and 0.32 mm inner diameter, coated with a 0.5-μm-thick film of BP20 (SGE, Australia), equivalent to Carbowax 20 M. Nitrogen was used as carrier gas at a flow rate of 1 ml/min.

Materials. Me_2S_2 and Pr_2S_2 were obtained from Merck. Dimethyl trisulfide (Me_2S_3) was supplied by Kodak. $MePrS_2$ was synthesized as previously described (Auger et al., 1989a). PCSO and MCSO were obtained by the hydrogen peroxide oxidation of *S*-propyl cysteine (PCS) and *S*-methyl cysteine (MCS), respectively (Barsley et al., 1964) and crystallization in a minimum volume of ethanol. PCSO: mp = 162°C. IR: 1020 cm^{-1} (s) characteristic of the S=O bond. MCSO: MP = 165°C. IR: 1030 cm^{-1} (s). MCS was obtained from Aldrich. PCS was prepared by alkylating cysteine with 1-bromopropane. Cystein was formed in situ by the reduction of cystine with sodium in liquid ammonia (Grenby and Young, 1960).

Trapping and Isolation. The headspace of volatile substances released at room temperature by frass in a closed 5-liter glass recipient was trapped for 6 hr on a cartridge (4 mm diameter, 20 mm long) packed with Tenax GC 60–80 mesh (30 mg). It was directly connected to a Gillian LFS 113 pump. Trapped substances were extracted with 1 ml of ether and injected.

A complete blank (trapping, extraction, and injection) preceded and followed each experiment.

Identification. Sulfur substances were identified by comparing their GC retention times to those of reference substances.

Breeding Larvae and Frass Collection. Frass was collected from *A. assectella* grown in 14:10 hr light–dark at constant 25°C on artificial corn flour-based food (Arnault, 1982). This diet (550 ml), in which about 400 larvae developed (number required for the production of the frass used in the experiments), was variously supplemented with 10 g of dry leeks, 0.1 mg of Pr_2S_2 or Me_2S_2, 0.15 g of PCSO or MCSO, the quantity of precursors present in leek (Bonnet et al., 1974).

RESULTS AND DISCUSSION

When larvae were fed an artificial diet without any supplement, no sulfur compounds were detected on gas chromatograms (Figure 1).

When the diet contained leek powder, the frass odor included Me_2S_2,

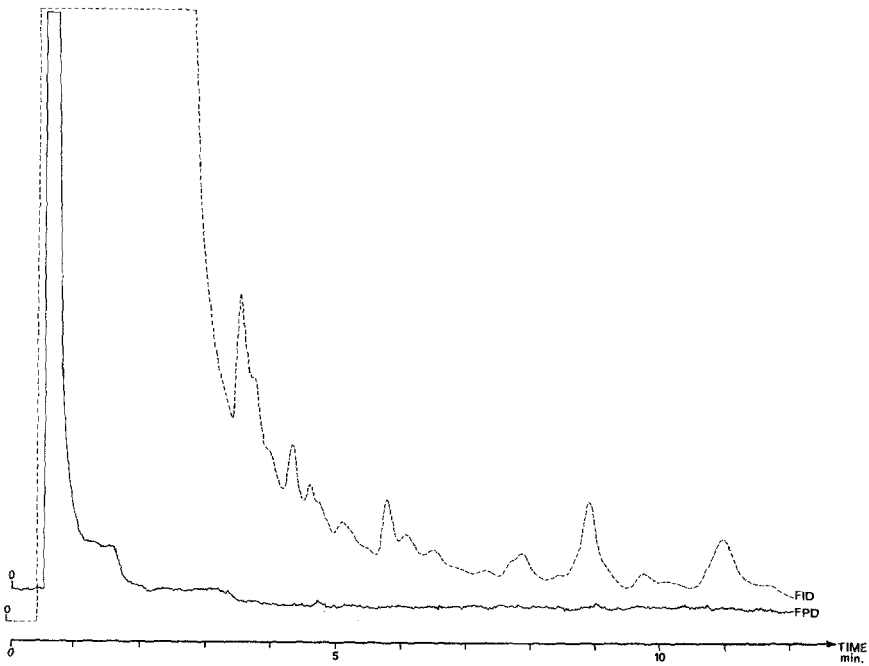

FIG. 1. Gas chromatogram of the leek moth frass volatiles trapped on Tenax, when larvae were fed on artificial diet without any supplement. Column: 25 m × 0.33 mm ID; temperature program: 1°C/min from 80°C to 150°C; on-column injection; FP and FI detectors.

$MePrS_2$, Me_2S_3, and a large majority of Pr_2S_2 (Figure 2). This chromatogram was very comparable to that obtained with frass of moths fed leek leaves (Auger et al., 1989b).

When the diet contained either PCSO or Pr_2S_2, frass odor included the same four substances as above, although the proportion of Pr_2S_2 was higher (Figure 3, Table 1). The addition of only propylthio compounds thus led to the appearance of methylthio compounds in the frass.

When the diet contained either MCSO or Me_2S_2, frass odor still included the same four substances as when the diet contained leek, but Pr_2S_2 was present in smaller proportions (Figure 4 and Table 1).

The comparison of experiments done with and without the addition of leeks shows that the host plant is responsible for the presence of each sulfur substance in the frass odor of moth larvae. Propylthio compounds are undoubtedly at the origin of the frass odor of larvae feeding on leeks, including methyl moiety disulfides, since the addition of PCSO or Pr_2S_2 is sufficient to make them appear

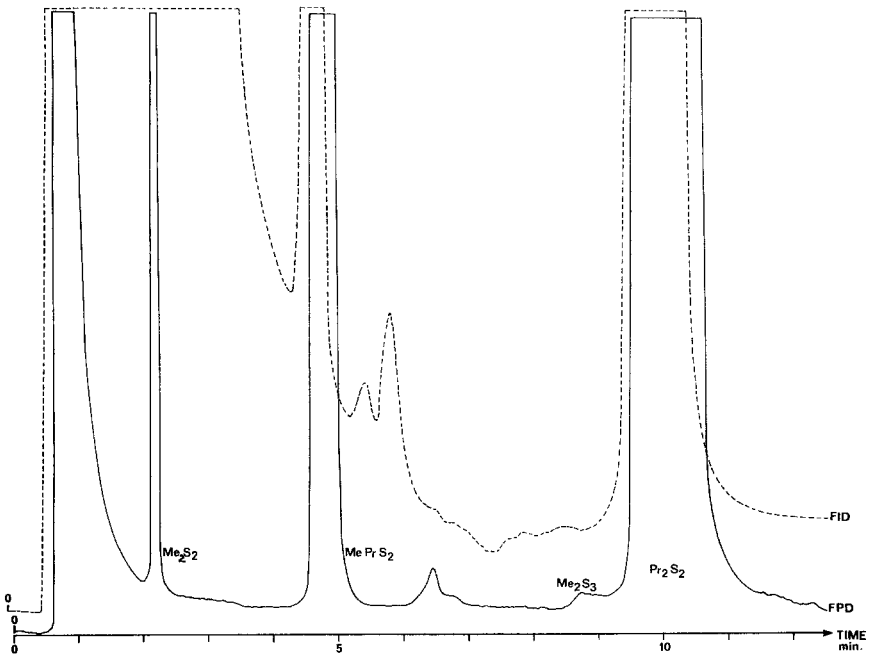

FIG. 2. Gas chromatogram of the leek moth frass volatiles trapped on Tenax, when larvae were fed on artificial diet containing leek powder. Column: 25 m × 0.33 mm ID; temperature program: 1°C/min from 80°C to 150°C; on-column injection; FP and FI detectors. Me_2S_2 = dimethyl disulfide; $MePrS_2$ = methyl propyl disulfide; Pr_2S_2 = dipropyl disulfide; Me_2S_3 = dimethyl trisulfide.

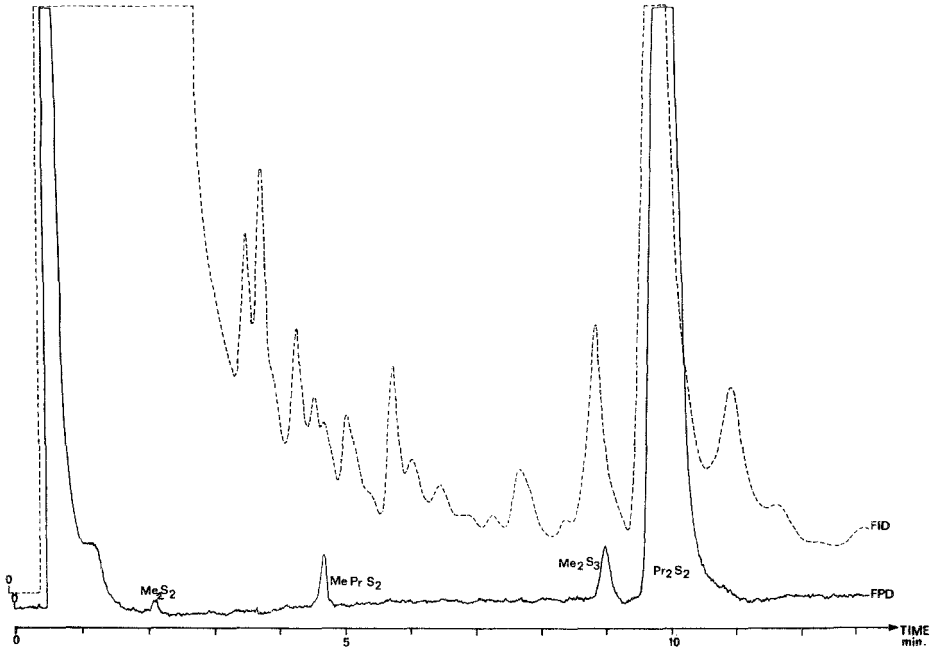

FIG. 3. Gas chromatogram of the leek moth frass volatiles trapped on Tenax, when larvae were fed on artificial diet containing either PCSO or Pr_2S_2 (here is the case of PCSO). Column: 25 m × 0.33 mm ID; temperature program: 1°C/min from 80°C to 150°C; on-column injection; FP and FI detectors. Me_2S_2 = dimethyl disulfide; $MePrS_2$ = methyl propyl disulfide; Pr_2S_2 = dipropyl disulfide; Me_2S_3 = dimethyl trisulfide.

TABLE 1. RELATIVE ABUNDANCE OF SULFUR SUBSTANCES IDENTIFIED IN FRASS VOLATILES OF *Acrolepiopsis assectella* FED ON ARTIFICIAL DIET VARIOUSLY SUPPLEMENTED[a]

	Sulfur substances[b]			
Diet additions	Me_2S_2	$MePrS_2$	Me_2S_3	Pr_2S_2
0	−	−	−	−
Leek	+	++	±	+++
MCSO[b]	+	++	++	+
Me_2S_2[b]	+	++	++	+
PCSO[b]	±	+	+	++++
Pr_2S_2[b]	±	+	+	++++

[a] The relative abundances are indicated by: ± < 1%; 1 < + < 10%; 10 < ++ < 60%; 60 < +++ < 90%; ++++ > 90%.

[b] Me_2S_2 = dimethyl disulfide; $MePrS_2$ = methyl propyl disulfide; Me_2S_3 = dimethyl trisulfide; Pr_2S_2 = dipropyl disulfide; MCSO = methyl cysteine sulfoxide; PCSO = propyl cysteine sulfoxide.

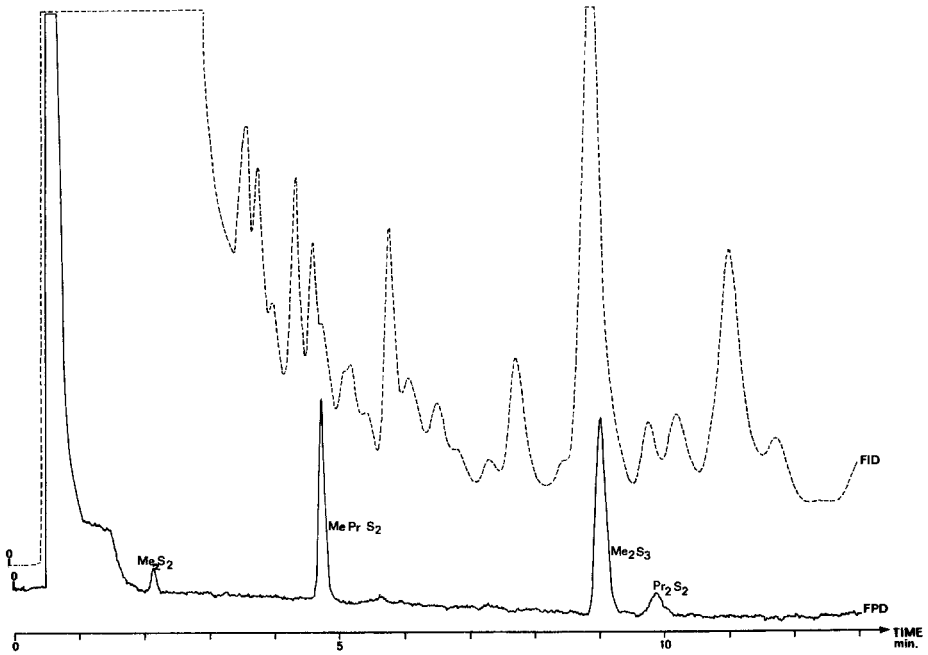

FIG. 4. Gas chromatogram of the leek moth frass volatiles trapped on Tenax, when larvae were fed on artificial diet containing either MCSO or Me_2S_2 (here is the case of MCSO). Column: 25 m × 0.33 mm ID; temperature program: 1°C/min from 80°C to 150°C; on-column injection; FP and FI detectors. Me_2S_2 = dimethyl disulfide; $MePrS_2$ = methyl propyl disulfide; Pr_2S_2 = dipropyl disulfide; Me_2S_3 = dimethyl trisulfide.

unexpectedly and in high proportions. It is equally surprising that the addition of methylthio compounds caused the appearance of Pr_2S_2 and $MePrS_2$.

These transformations from an *S*-propyl to an *S*-methyl moiety, and vice versa, could arise by improbable modifications of the carbon chain. Nevertheless, during degradation of the precursor to disulfide in onions by soil microorganisms, *S*-1-propenyl moiety is transformed to *S*-propyl moiety (Ikeshoji, 1984).

It is more reasonable to believe in a radical exchange between the substance added to the diet and another compound, either sulfur or not, in the diet. This exchange probably occurs under the effect of enzymes remaining to be determined that could exist in the nutrient medium or the digestive tract of the larvae.

In light of the results, totally comparable when precursors or disulfides are added, two cases are possible:

1. The exchange occurs only at the level of disulfides and the precursors decompose into disulfides with the same radical in the larval digestive tract, analogously to that suggested by the odor of leeks, or else by microorganisms.

A number of microorganisms transform *S*-alkyl cysteines and *S*-alkyl cysteine sulfoxides into disulfides. This is the case of soil bacteria (King and Coley-Smith, 1969) and *E. coli*-type intestinal bacteria (Virtanen, 1965).

2. The exchange occurs at the levels of both precursors and disulfides.

Concerning the precursors, Cherest et al. (1970) showed that *Saccharomyces cerevisiae* exchanged methyl for ethyl on MCS in presence of ethionine (*S*-ethyl homocysteine). In the case of the addition of PCSO, an analogous mechanism would exchange the propyl moiety for methyl via, e.g., methionine, which is always present. In the case of the addition of MCSO, it is necessary to invoke the more hypothetical intervention of an *S*-propyl amino acid, present in the artificial diet lacking *Allium*.

Several other transformations from one alkyl to another have been reported when *S*-alkyl cysteines are transformed into disulfides and sulfides; thus, in presence of thiamine (vitamin B_1), identified in almost all living tissues, a *Bacillus subtilis* enzyme decomposes MCSO into the mixed methyl and thiamine disulfide (Murakami, 1960). Similarly, the fungus *Scopulariopsis brevicaulis* transforms *S*-alkyl cysteines (R = Me, Et, *n*-Pr, allyl) into mixed alkyl and methyl sulfides (Challenger and Charlton, 1947). The radical exchanged can also be propyl or methyl, rather than *S*-propyl or *S*-methyl, and arise from one of the numerous constituents in the nutritive diet. For example, the decomposition of ethionine in presence of pectin leads to the mixed methyl and ethyl sulfide (Casey et al., 1963).

These hypotheses can also be invoked for the exchange of ingested disulfides or those arising from the breakdown of precursors. We are unaware of any observations that enable a mechanism for this breakdown to be proposed.

Regardless, the substances emitted in the feces are the same as those emitted in the decomposition of root emanations from onions (Ikeshoji, 1984). The proportion of Me_2S_2 and $MePrS_2$ in relation to Pr_2S_2 is much higher than that observed in the odor of onion. The explanation furnished by Ikeshoji (1984) is not applicable to our case, because the *S*-propyl moiety would arise from *S*-propenyl-1-cysteine sulfoxide, while the *S*-methyl moiety would arise from glutamyl-*S*-methyl cysteine; both are specific to *Allium* and absent in the experiments carried out in the absence of leek.

No decisive element enables either of these hypotheses to be supported for the moment. The microbiological study of the digestive tract of leek moths may lead to the proposal of a mechanism for these unexpected transformations. They may involve precursor biosynthesis pathways in *Allium*, starting with cysteine in the artificial diet.

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SUSCEPTIBILITY OF *Heliothis zea* (BODDIE) LARVAE TO *Nomuraea rileyi* (FARLOW) SAMSON
Effects of α-Tomatine at the Third Trophic Level

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Abstract—To determine the impact of α-tomatine at the third trophic level, the following model was developed: *Nomuraea rileyi* (Farlow) Samson, the secondary consumer, acting on *Heliothis zea* (Boddie), the primary consumer, fed an artificial diet modified with α-tomatine. In vitro, the allelochemical inhibited colony formation and growth of the fungus. The in vivo test revealed that larval growth and developmental time were affected by α-tomatine and *N. rileyi*. Detrimental effects on pupal development were observed in larvae fed diet containing α-tomatine and also treated with *N. rileyi* (LC_{90}). The fungus was detected in the hemolymph and tissue of larvae treated with two lethal concentrations (LC_{50} and LC_{90}) of *N. rileyi*, including those fed α-tomatine. At the LC_{50}, α-tomatine protected larvae against *N. rileyi* and increased survivorship; at the LC_{90}, it inhibited the development of *N. rileyi*, thereby reducing production of conidia. Thus, the allelochemical α-tomatine retains its antifungal qualities beyond the second trophic level, inhibiting the development of *N. rileyi* in *H. zea*.

Key Words—*Heliothis zea*, Lepidoptera, Noctuidae, *Nomuraea rileyi*, Deuteromycotina, fungi, α-tomatine, allelochemical, third trophic level.

INTRODUCTION

α-Tomatine, a secondary plant product, is a steroid glycoalkaloid that is found in large quantities in all parts of *Solanum* and *Lycopersicon* plants except in dormant seeds and in ripe tomato fruits (Roddick, 1974; Schlosser, 1975). The allelochemical is toxic to a wide range of living organisms, including fungi

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(Défago and Kern, 1983). α-Tomatine has detrimental effects at the second trophic level (plant–disease or plant–insect) (Arneson and Durbin, 1968; Défago and Kern, 1983; Duffey et al., 1986) as well as on parasitoids (third trophic level) (Thurston and Fox, 1972; Campbell and Duffey, 1981; Barbosa et al., 1982; Barbosa and Saunders, 1984).

α-Tomatine is toxic to many fungi in vitro (Défago and Kern, 1983; Costa and Gaugler, 1989). There is some evidence that toxicity is due to surfactant effects on cell membranes and membrane destabilization, resulting primarily from complex formation between the glycoside and membrane sterols. α-Tomatine also causes leakage from and/or impairment of fungal hyphae, higher plant cells, and plant cell organelles that contain little or no cholesterol (Roddick, 1979).

Hare and Andreadis (1983) found that when the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), was fed tomato, *Lycopersicon esculentum* Miller, foliage, the susceptibility to infection by the fungus, *Beauveria bassiana* (Balsamo) Vuillemin, was decreased. They suggested that the α-tomatine was implicated in the reduced susceptibility to *B. bassiana* infection.

Apparently, there have been no studies reported to determine the direct effects of α-tomatine beyond the second trophic level, with an entomopathogenic fungus as secondary consumer (third trophic level). To examine such a system, the following model was used: *Nomuraea rileyi* (Farlow) Samson, the secondary consumer, acting on the corn earworm, *Heliothis zea* (Boddie), the primary consumer, fed an artificial diet modified with a secondary plant compound (α-tomatine).

METHODS AND MATERIALS

Heliothis zea larvae were reared on diet developed by Burton (1969) and modified with α-tomatine (United States Bio-chemicals Corp., Cleveland, Ohio). *Nomuraea rileyi* was originally isolated from soybean looper, *Pseudoplusia includens* (Walker), larvae collected in 1988 from soybean fields near St. Gabriel, Louisiana. *N. rileyi* cultures were grown on Saboraud maltose agar + 2% yeast extract (SMA+Y) at 23.8°C, 80% relative humidity, and 14:10 hr light–dark photoperiod. Two lethal concentrations (LC_{50} = 30 conidia/mm^2 and LC_{90} = 10^3 conidia/mm^2) of conidia, determined from preliminary data (unpublished), were used for conducting the in vivo test.

In Vitro Test. Conidia from the cultures were suspended in 0.008% Triton X-100 (Rohm and Haas Co., Philadelphia, Pennsylvania) in sterile distilled water. Conidia were concentrated to 8.5×10^7 spores/ml, as determined with a Petroff-Hausser counting chamber. The α-tomatine was diluted to test concentrations (Table 1) in sterile distilled water. Test concentrations utilized are

TABLE 1. IN VITRO EFFECTS OF α-TOMATINE ON DEVELOPMENT OF *Nomuraea rileyi*

Treatments	Diameters (mm) of zones inhibited[a]
Negative control[b]	0 d
Positive control[c]	45.4 ± 1.60 a
α-Tomatine (%)	
0.001	0 d
0.005	0 d
0.010	0 d
0.050	18.9 ± 0.80 c
0.100	21.7 ± 1.32 b

[a]Means ± SE followed by the same letter are not significantly different [$P > 0.05$, Duncan's multiple-range test (SAS Institute, 1985)].
[b]Sterile distilled water.
[c]Mancozeb (Dithane F 45, at recommended rate).

within the range for α-tomatine levels found in tomato plants (Duffey et al., 1986; Roddick, 1974).

The SMA+Y medium was poured into 100 × 15-cm sterile disposable plastic Petri plates and allowed to solidify and cool. Then 0.2 ml of the stock *N. rileyi* suspension was added to the center of each plate. Each plate was rotated to spread the suspension across the plate. After 1 hr, one sterile, 6-mm-diameter, filter paper disk (Whatman No. 1) was dipped in a treatment and placed in the center of the plate. Two controls were used: a positive control, in which the disk was dipped in the fungicide mancozeb (Dithane F45, Rohm and Haas) at its recommended rate; and a negative control, in which the disk was dipped in sterile distilled water. The plates were held at 23.8°C, 80% relative humidity, and 14 : 10 hr light–dark photoperiod for five days.

Each treatment (concentration of the α-tomatine) and the controls were replicated seven times. Diameters of the zones of inhibited fungal growth were measured on day 5 after treatment. Data were subjected to analysis of variance (ANOVA) and Duncan's multiple range test (SAS Institute, 1985).

In Vivo Test. α-Tomatine was added at the time of the diet preparation at 0.9 μmol/g of wet weight of diet. Duffey et al. (1986) demonstrated that this dosage of α-tomatine caused 50% reduction in *H. zea* larval growth (ED_{50}) when compared with a control.

The following treatments were established: α-tomatine + *N. rileyi* LC_{50} (α-t+N50), α-tomatine + *N. rileyi* LC_{90} (α-t+N90), α-tomatine (α-t), control + *N. rileyi* LC_{50} (C+N50), control + *N. rileyi* LC_{90} (C+N90), and control (C).

Two batches of diet were prepared. The first batch was modified with α-tomatine, while the second batch was not (control). One neonate of *H. zea* was placed in each diet cup (29.6 ml) and reared in an environmental chamber at 27°C and 14 : 10 hr light–dark photoperiod. Each batch of insects was divided into three groups (100 diet cups each). Two of the groups were treated with *N. rileyi* conidia, and the other remained as a group control.

The two lethal concentrations (LC_{50} = 30 conidia/mm^2 and LC_{90} = 10^3 conidia/mm^2) of conidia were spread over SMA+Y plates and allowed to dry for 1 hr (Boucias et al., 1984). Ten second-instar larvae of *H. zea* were introduced per plate. Plates were placed in an environmental chamber at 25°C, 14 : 10 hr light–dark photoperiod. After 24 hr, the larvae were returned to diet cups with or without α-tomatine, depending on the treatment. Larvae in the control were handled in the same manner but were not exposed to the entomopathogen.

Treatments were arranged in a completely randomized design (CRD) and placed in an environmental chamber at 25°C, the optimal temperature for the development of *N. rileyi* (Gardner, 1985), and at 14 : 10 hr light–dark photoperiod. High humidity (>80%) was maintained inside each cup by placing a few drops of water twice a day in the cup cap during the first two days after infection. This procedure was employed because penetration of the cuticle of the larvae by the *N. rileyi* germ tube is enhanced by high humidity and occurs within two days of topical treatment (Mohamed et al., 1978).

At four days after exposure to the fungal conidia, 10 larvae from each treatment were selected at random, dissected, and checked for fungal hyphal bodies under phase microscopy. This was done to determine if α-tomatine permitted early fungal development but inhibited later growth.

Larval weights at 10 days after treatment (when larvae in control reached fifth instar) and daily observations of symptoms or signs of the disease were recorded. Data on pupal weight and larval development also were recorded. Mortality was recorded daily until pupation. To confirm the cause of death, cadavers were placed in Petri dishes with moistened filter paper at 25°C to allow for external fungal growth and sporulation.

Tests for normality of the data were performed. In cases in which the data did not follow a normal distribution, a log transformation was performed prior to statistical analysis. Data on percent mortality were subjected to arc-sin transformations. Data were analyzed by the general linear model of the statistical analysis system (SAS Institute, 1985), and Duncan's multiple-range test was used to identify significant differences ($P \leq 0.05$) among treatment means.

RESULTS AND DISCUSSION

In Vitro Test. α-Tomatine at the higher concentrations (0.05 and 0.10% wet wt) inhibited growth of *N. rileyi* (Table 1). The measurements (diameters) of zones of inhibition were significantly different (F = 238.2; df = 12, 36; P

≤ 0.05) among treatments. Five days after treatment, the positive control (the fungicide, mancozeb) gave the highest zone of inhibition, followed by α-tomatine at 0.05 and 0.10% (Table 1). These data showed the direct inhibition of *N. rileyi* by α-tomatine.

Similarly, Costa and Gaugler (1989) demonstrated in vitro the inhibition of α-tomatine against the entomopathogen *B. bassiana*. Further studies would be necessary to understand the mode of action of α-tomatine against *N. rileyi*.

In Vivo Test. Both the allelochemical α-tomatine and the entomopathogen *N. rileyi* had a detrimental effect on growth of *H. zea* larvae (Table 2). Significantly lower larval weights ($F = 15.55$; $df = 5, 141$; $P \leq 0.05$) were observed in all treatments when compared with the control. No significant differences in larval weights ($P > 0.05$) were found between treatments in which the larvae were subjected to the LC_{50} or LC_{90} dosages of fungal conidia. However, the combination of α-tomatine and the LC_{90} dosage had a significantly ($P \leq 0.05$) greater detrimental effect on larval weight than the combination of α-tomatine and the LC_{50} dosage.

α-Tomatine significantly ($P \leq 0.05$) reduced larval growth independently of the presence of *N. rileyi*, resulting in a 58.5% decrease in larval weight relative to the control (normal diet devoid of α-t) (Table 2). Similar results were obtained by Duffey et al. (1986), who found that α-tomatine served as a growth-reducing antibiotic for *H. zea* larvae. The treatments α-t+N50 and α-t+N90 caused 52.9% and 20.9% relative growth, respectively, whereas the treatments C+N50 and C+N90 demonstrated 54.61% and 27.1% relative growth, respectively (Table 2).

Among larvae that pupated, there were no significant differences ($F = 2.46$; $df = 3, 32$; $P > 0.05$) in pupal weight (Table 2). However, detrimental effects on pupal development were observed when α-tomatine was combined

TABLE 2. EFFECTS OF α-TOMATINE AND *Nomuraea rileyi* ON GROWTH OF *Heliothis zea*

Treatments[a]	Mean body weight (mg)[b]	
	10-day-old larvae	Pupae
α-t + N50	175.6 ± 18.7b	366.3 ± 12.4a
C + N50	181.3 ± 20.5b	358.3 ± 15.2a
α-t + N90	69.5 ± 33.3c	[c] [c]
C + N90	90.0 ± 55.3bc	355.9 ± 16.8a
α-t	137.8 ± 18.4bc	369.9 ± 11.2a
C	331.9 ± 19.5a	402.8 ± 11.8a

[a] α-t = α-tomatine, C = control, N50 = *N. rileyi* LC_{50}, N90 = *N. rileyi* LC_{90}.
[b] Means ± SE followed by the same letter within columns are not significantly different [$P > 0.05$, Duncan's multiple-range test (SAS Institute, 1985)].
[c] No pupation occurred in this treatment.

with the LC_{90} dosage of *N. rileyi* conidia. Larvae in that treatment were unable to pupate.

Larval development time was increased by the effects of α-tomatine and also by *N. rileyi*. Significant differences ($F = 40.24$; $df = 3, 107$; $P \leq 0.05$) in larval development times were observed in all treatments containing the allelochemical when compared with treatments devoid of it. Treatments α-t+N50, α-t+N90, and α-t increased larval development time by 1.96, 2.75, and 4.00 days, respectively, when compared with treatments C+N50, C+N90, and C (Table 3). The larvae exposed to conidia of *N. rileyi* not showing symptoms of infection also exhibited increased development times. The increased larval development times associated with α-tomatine, and, to a lesser extent *N. rileyi*, gave the larvae more time to feed on the diet and reach the critical body weight (Wigglesworth, 1972) necesssary to initiate pupation. Undoubtedly, this factor explains the lack of differences among treatments in pupal weights (Table 2).

Data on the effects of α-tomatine and *N. rileyi* on the mortality of *H. zea* larvae are shown in Table 4. Significantly ($F = 15.91$; $df = 5, 18$; $P \leq 0.05$) less mortality (60.4%) occurred in treatment α-t+N50 than in C+N50. Although no significant difference ($P = 0.06$) in mortality occurred between α-t+N90 and C+N90, mortality was 18.0% less in the treatment containing α-tomatine. No significant differences ($P > 0.05$) in mortality were evident between the treatments α-t+N50 and α-t or between α-t and the control. These data indicate that the allelochemical reduces the mortality associated with *N. rileyi* infection.

Dissections of larvae, under phase microscopy, from treatments exposed to the entomopathogen revealed the presence of fungal hyphal bodies in all larvae examined. Larval cadavers from treatments α-t+N50, C+N50,

TABLE 3. EFFECTS OF α-TOMATINE AND *Nomuraea rileyi* ON DEVELOPMENT OF *Heliothis zea*

Treatments[a]	Time to pupation (days)[b]
α-t + N50	18.7 ± 0.29a
C + N50	16.7 ± 0.38b
α-t + N90	19.0 ± 0.37a
C + N90	16.2 ± 0.28b
α-t	19.6 ± 0.25a
C	15.6 ± 0.26c

[a] α-t = α-tomatine, C = control, N50 = *N. rileyi* LC_{50}, N90 = *N. rileyi* LC_{90}.
[b] Means ± SE followed by the same letter are not significantly different [$P > 0.05$, Duncan's multiple-range test (SAS Institute, 1985)].

TABLE 4. EFFECTS OF α-TOMATINE AND *Nomuraea rileyi* ON MORTALITY OF *Heliothis zea*

Treatments[a]	Mortality (%)[b]	Sporulated (%)[c]
α-t + N50	24.7 ± 6.1c	11.2 ± 5.4b
C + N50	62.5 ± 5.5b	35.5 ± 5.5a
α-t + N90	70.0 ± 6.1ab	2.0 ± 6.0b
C + N90	87.5 ± 6.0a	42.5 ± 6.0a
α-t	8.0 ± 5.4cd	
C	5.0 ± 6.0d	

[a] α-t = α-tomatine, C = control, N50 = *N. rileyi* LC_{50}, N90 = *N. rileyi* LC_{90}.
[b] Means ± SE followed by the same letter are not significantly different [$P > 0.05$, Duncan's multiple-range test (SAS Institute, 1985)].
[c] Percent of cadavers that produced fungal conidia.

α-t+N90, and C+N90 sporulated as a result of *N. rileyi* infection (Table 4). A significant decrease ($F = 13.06$; $df = 5, 18$; $P \leq 0.05$) in percent sporulation due to *N. rileyi* infection was obtained when treatment α-t+N90 was compared with C+N90 and when α-t+N50 was compared with C+N50, with 95.3% and 68.3% less larvae exhibiting *N. rileyi* sporulation, respectively, associated with treatments that contained the allelochemical. No significant differences ($P > 0.05$) in percent sporulation were observed between treatments α-t+N50 vs. α-t+N90 or C+N50 vs. C+N90.

Only 2.0% of the larvae in treatment α-t+N90 demonstrated sporulation of *N. rileyi*, thereby confirming infection with the entomopathogen (Table 4). However, most of the larval mortality that occurred in this treatment did so during the first three days after inoculation, with none of the larvae reaching the third instar. These results indicate that the high concentration (LC_{90}) of conidia in combination with α-tomatine increased the stress on the larvae, accelerating death without ample time for development of the fungus. In the C+N90 treatment, mortality occurred later in the development of the larvae, and the fungus was able to develop (42.5% sporulation).

These results indicate that the presence of α-tomatine in the diet of *H. zea* larvae was able to inhibit the development of *Nomuraea rileyi* conidiophores and conidia, especially at higher doses (LC_{90}) of conidia. At that level, the allelochemical, although not affecting the total percent mortality, did decrease the percent of larvae producing conidia (Table 4). This condition could hamper an epizootic of the disease under field conditions because of decreased inoculum. At the LC_{50} dosage, α-tomatine significantly ($P \leq 0.05$) decreased the mortality of *H. zea* larvae when compared with the larvae that fed on diet devoid of the allelochemical (Table 4). Thus, at low levels of inoculum, α-tomatine has the potential to protect the host against *N. rileyi*. This condition is of adap-

tative advantage for insect herbivores that can feed on plants producing allelochemicals and then gain protection from the entomopathogen.

These results closely parallel those found in studies on *Manduca sexta* (L.) (Krischik et al., 1988). Higher levels of the allelochemical nicotine increased the survivorship of *M. sexta* when fed diet with *Bacillus thuringensis* var. *kurstaki* Berliner (BT). They suggested that plant allelochemicals are important factors in reducing larval mortality caused by BT on certain crops. Ramoska and Todd (1985) also suggested that the presence of a plant-produced fungal inhibitor protected *Blissus leucopterus* (Say) against the entomogenous fungus *Beauveria bassiana* (Bals.) when fed sorghum and corn.

Hare and Andreadis (1983) implied that α-tomatine in tomato foliage was responsible for the reduced susceptibility of the Colorado potato beetle to *B. bassiana*. Our data clearly demonstrated that α-tomatine inhibited development of another entomogenous fungus, *N. rileyi*, thus supporting their supposition.

In summary, we conclude that the allelochemical α-tomatine retains its antifungal qualities beyond the second trophic level, inhibiting the development of *N. rileyi* in *H. zea*. Whether the effects are mediated directly upon the fungus or indirectly via stress on the host remains to be determined. Different responses were obtained at the two conidia concentrations. At the LC_{50}, α-tomatine has the potential to protect against the fungus *N. rileyi* and increase survivorship of the larvae, and at the LC_{90}, it is able to inhibit the development of *N. rileyi*, thereby reducing production of conidia. Therefore, in addition to many other environmental factors that affect the expression of an entomopathogen under field conditions (Carruthers and Soper, 1987), allelochemical effects through the trophic levels need to be considered, as suggested by Duffey et al. (1986).

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PERFLUORINATED MOTH PHEROMONES
Synthesis and Electrophysiological Activity

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Abstract—Perfluoroalkyl analogs of pheromone constituents were synthesized and responses from male antennal olfactory receptor neurons from three moth species were recorded during stimulation by these analogs. In each analog, the hydrophobic terminus, either a butyl or hexyl substituent on the (Z)-alkenyl chain, was replaced with a perfluorobutyl (Pfb, C_4F_9) or perfluorohexyl (Pfh, C_6F_{13}) moiety. Perfluoroalkyl analogs were more volatile than their hydrocarbon analogs, showing a decrease in gas chromatographic retention time by two to four methylene equivalents (Kovàts retention indices). Specialist neurons of male *Heliothis zea* responded to a 0.02-μg dose of (Z)-11-hexadecenal (Z11-16:Al) and a dose of 200 μg of Pfb-Z11-16:Al with similar spike discharge rates. The HS(a) neurons of *Trichoplusia ni* responded to a dose of 0.02 μg of Z7-14:OAc and a dose of 10 μg of Pfb-Z7-12:Ac with similar discharge rates. The same difference in sensitivity to Pfb-Z7-12:OH and Z7-12:OH was observed for the responses of the HS(b) neuron and for the responses of the NS(a) neurons to Pfb-Z9-14:Ac and Z9-14:Ac. Sensilla of *Diatraea grandiosella* similarly showed 100- to 1000-fold greater sensitivity to Z9-16:Al and Z11-16:Al than to Pfh-Z9-16:Al and Pfb-Z11-16:Al. Thus, replacement of terminal alkyl groups with perfluoroalkyl groups in pheromone components produced biologically active compounds with increased volatility and displaced electrophysiological response profiles.

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Because of the diminished receptor cell sensitivity, we suggest that the binding of the fluorinated analogs to a putative receptor is reduced as a result of less favorable interaction between the hydrophobic protein binding site and the more rigid and more polar perfluoroalkyl moiety.

Key Words—Fluorination, perfluoroalkyl, pheromone analog, Lepidoptera, *Diatraea grandiosella*, *Heliothis zea*, *Trichoplusia ni*, single-cell recording, electrophysiology, receptor binding.

INTRODUCTION

Female moths produce blends of simple saturated and unsaturated fatty alcohols, acetates, and aldehydes that act as sex attractant pheromones for males (Tamaki, 1985). A number of monofluorinated pheromone analogs (Prestwich, 1986; Prestwich et al., 1988; McLean et al., 1989) and selectively fluorinated pheromone mimics (Briggs et al., 1986) have been prepared for previous studies on the molecular basis of pheromone perception and metabolism (Prestwich, 1987a,b; Prestwich and Streinz, 1988). In addition, Camps and coworkers (1986) also have synthesized alkenyl fluorides as pheromone analogs. However, none of these studies has addressed the profound effects on chemical and physical properties of pheromone components that would result from polyfluorination of the alkyl chain. Such polyfluoroalkyl analogs would allow study of the relative importance of ligand hydrophobicity and polarity in receptor binding to antennal transductory proteins (Vogt, 1987; Kaissling, 1986). We describe herein the preparation of analogs of several pheromone constituents in which perfluorobutyl or perfluorohexyl chains are substituted for the normal hydrocarbon termini.

The compounds examined have significance to pheromone-elicited behaviors and selective receptor neuron responses (Mayer and Mankin, 1985) in two noctuid and one pyralid pest species. First, we selected the corn earworm moth *Heliothis zea* (Boddie) (Noctuidae), which has (Z)-11-hexadecenal as a major component in the pheromone blend (Klun et al., 1980). Second, responses from the cabbage looper *Trichoplusia ni* (Hübner) (Noctuidae) were obtained to (Z)-7-dodecenyl acetate and (Z)-9-tetradecenyl acetate, two of the major pheromone components in a redundant blend (Linn et al., 1984). Third, we chose the southwestern corn borer, *Diatraea grandiosella* (Dyer) (Pyralidae), which employs (Z)-11-hexadecenal and (Z)-9-hexadecenal as pheromone components (Hedin et al., 1986). Based on these data, we suggest a model that correlates biological responses with altered physicochemical properties of the pheromone analogs.

METHODS AND MATERIALS

Synthesis of Polyfluoroalkyl Analogs

General. 3-Octyn-1-ol (I), 3-decyn-1-ol (II), and 7-dodecyn-1-ol (III) were purchased from Farchan Labs. Solvents were distilled before use. Anhydrous tetrahydrofuran (THF) and diethyl ether were distilled from sodium benzophenone ketyl prior to use. Flash column chromatography was carried out using Woelm silica gel (32–63 μm). Thin-layer chromatography (TLC) was performed using MN Polygram Sil G/UV254 silica gel plates (4 cm × 8 cm × 0.25 mm). The developed TLC plates were visualized by staining with 3% vanillin (w/v) in ethanol containing 0.3% sulfuric acid or with 10% phosphomolybdic acid (w/v) in ethanol. Proton nuclear magnetic resonance ([^{1}H]NMR) spectra were obtained in $CDCl_3$ solution using a QE-300 spectrometer and 0.03% tetramethylsilane (TMS) as an internal standard; chemical shifts (δ) are expressed as parts per million downfield from TMS. [^{19}F]NMR spectra were obtained on a Nicolet NT-300 spectrometer using trichlorofluoromethane ($CFCl_3$) as an internal standard in benzene-d_6; chemical shifts (ϕ) are expressed as parts per million upfield from $CFCl_3$. Mass spectra (MS), as well as high-resolution mass spectra (HRMS) (70 eV, electron impact), were obtained using a Spectros MS 30 spectrometer with a DS 50 data system. Gas chromatography was carried out on a Varian model 3700 GC equipped with a fused silica capillary column (DB-1 or DB-5, 30 m × 0.263 mm, 0.25-μm film thickness). All glassware, syringes, and needles were dried in an oven at 110°C before use. The glassware was assembled hot and cooled under a flow of dry nitrogen. All of the reactions were carried out under a small positive pressure of dry nitrogen.

The synthetic procedures below are illustrative of the methods used (1) to convert commercially available 3-alkyn-1-ols and 7-dodecyn-1-ol to ω-alkyn-1-ols, (2) to effect free-radical addition of perfluorobutyl iodide (PCR Chemicals) or perfluorohexyl iodide (Japan Halon Co.) to the alkene, (3) isolation of pure (*E*)-alkenyl iodides and their conversion to (*Z*)-alkenes, and (4) functional group modifications to produce the perfluoroalkyl-modified analogs of pheromone alcohols, acetates, and aldehydes for three moth species.

9-Decyn-1-ol (V). This was obtained from 3-decyn-1-ol (II) in >90% yield using the acetylene zipper reaction as described by Abrams and Shaw (1987). [^{1}H] NMR δ 6.663 (t, J = 6.2 Hz, H-1), 2.217 (tt, J = 6.8, 2.6 Hz, H-8) 1.950 (t, J = 2.6 Hz, H-10), 1.65 (m, H-2 to H-7).

9-Iodo-11,11,12,12,13,13,14,14,14-nonafluorobutyltetradec-9-en-1-ol (VIII). 9-Decyn-1-ol (V) (462 mg, 3 mmol), *n*-perfluorobutyl iodide (1.56 g, 4.5 mmol), and azobis(isobutyronitrile) (AIBN, 50 mg, 0.3 mmol) were placed

in a heavy-walled glass tube (2.5 cm OD, 1.8 cm ID × 20 cm, with a narrow neck 5 cm from bottom) equipped with a magnetic stir bar. The mixture was frozen (liquid N_2, 77°K), degassed, and thawed under nitrogen atmosphere to eliminate oxygen. This process was repeated again. Then the tube was sealed under vacuum while the contents were still frozen. The mixture was slowly warmed to room temperature and heated to 80°C for 18 hr. After cooling down with liquid nitrogen, the tube was opened. The crude product was purified and the *E* and *Z* isomers were separated by flash column chromatography (hexane–ethyl acetate, 10:1). Fractions with over 98% isomeric purity (GC) were combined; the remaining fractions were pooled, concentrated, and rechromatographed. After two chromatographic separations, two portions were obtained, isomeric pure *E* isomer of Pfb-9-iodo-Δ9–14:OH (VIII) (750 mg, 50% yield) and a mixture of *E* and *Z* isomers of alkenyl iodide VIII (495 mg, 33% yield). A small aliquot was analyzed by GC, which indicated that before chromatography the *E–Z* ratio was 8.7:1. [^{1}H]NMR δ 6.337 (t, J = 14.5 Hz, H-10), 3.620 (t, J = 6.30 Hz, H-1), 2.609 (t, J = 6.55 Hz, H-8), 1.59 (br s, H-2, H-7), 1.30 (br s, H-3 to H-6); [^{19}F]NMR ϕ 79.90 (tt, J = 12.36, 2.70 Hz, F-14), 104.06 (m, 2F), 122.79 (m, 2F) 124.46 (m, 2F).

The other three perfluoroalkyl alkenyl iodides were prepared in an analogous fashion. The yields and *E–Z* ratios were: (1) 11-iodo-Pfb-Δ 11–16:OH (IX), 80% yield, *E/Z* = 9.2; (2) 7-iodo-Pfb-Δ7–12:OH (VII), 84% yield, *E/Z* = 7.3; (3) 9-iodo-Pfh-Δ9–16:OH (X) = 80% yield, *E/Z* = 7.0.

(Z)-11,11,12,12,13,13,14,14,14-Nonafluorotetradec-9-en-1-ol (Pfb-Z9–14:OH) (XII). A solution of *n*-BuLi (1.6 M soln. in hexane, 3 ml) was added to a solution of Pfb-alkenyl iodide VIII (472 mg, 1 mmol) in 10 ml of dry ether at −78°C. The mixture was stirred for 30 min and quenched with precooled (−78°C) methanol (3 ml). After warming to room temperature, the mixture was poured into 20 ml of NH_4Cl solution and extracted with ether (3 × 15 ml). The combined ether extracts were washed with brine (1 × 10 ml), dried ($MgSO_4$), concentrated in vacuo, and chromatographed (hexane–ethyl acetate, 10:1) to yield 300 mg (83%) of Pfb-Z9–14:OH (XII). [^{1}H]NMR δ 6.117 (dtt, $^3J_{H9,H10}$ = 12.0 Hz, $^3J_{H9,H8}$ = 7.8 Hz, $^4J_{H9,F11}$ = 2.4 Hz, H-9), 5.472 (m, H-10), 3.625 (t, 6.1 Hz, H-1), 2.294 (br s, H-8), 1.61 (m, H-2, H-7), 1.30 (br s, H-3 to H-7). [^{19}F]NMR ϕ 80.84 (t, F-14), 105.79 (m, 2F), 123.99 (m, 2F), 125.24 (m, 2F). FT-IR (neat): 3333.2 br, 2930.9, 2858.5, 1663.0, 1353.5, 1235.7, 1133.6, 878.4, 742.7 cm^{-1}.

Pfb-Z9–14:Al (XV). A mixture of Pfb-Z9–14:OH (XII) (38 mg, 0.1 mmol), 4 Å molecule sieves (10 mg), and 4-methylmorpholine *N*-oxide (NMO, 27 mg, 0.15 mmol) in 2 ml of dry methylene chloride was stirred at 25°C. After 10 min, tetrapropylammonium perruthenate (TPAP, 0.5 mg) was added to the mixture. The color of the solution slowly changed from light green to dark brown; TLC showed the reaction was completed after another 10 min. The

mixture was filtered through silica gel, concentrated in vacuo, and purified by flash chromatography (hexane–ethyl acetate, 20:1) to yield 31 mg (81%) of Pfb-Z9–14:Al (XV). [^{1}H]NMR δ 9.772 (t, J= 1.22 Hz, H-1), 6.094 (dtt, $^3J_{H9,H10}$ = 12.10 Hz, $^3J_{H9,H8}$ = 7.69 Hz, $^4J_{H9,F11}$ = 2.30 Hz, H-9), 5.493 (m, H-10), 2.472 (td, J = 7.32, 1.20 Hz, H-2), 2.36 (br s, H-8), 1.61 (m, 1.35 br s, 8H); [^{19}F]NMR was unchanged from that for Pfb-Z9–14:OH.

Pfb-Z7–12:Ac (XVIII). A mixture of Pfb-Z7–12:OH XI (35 mg, 0.1 mmol) (prepared analogously to Pfb-Z9–14:OH), acetic anhydride (36 mg, 0.3 mmol), and 4-dimethylaminopyridine (DMAP, 5 mg) in 2 ml of pyridine was stirred at room temperature for 3 hr. The mixture was diluted with 30 ml of hexane–ethyl acetate (7:3) solution, washed with saturated $CuSO_4$ solution (3 × 1 ml), dried ($MgSO_4$), concentrated in vacuo, and chromatographed (hexane–ethyl acetate, 50:1) to yield 36 mg (92%) of Pfb-Z7–12:Ac (XVIII). [^{1}H]NMR δ 6.127 (dtt, $^3J_{H7,H8}$ = 12.05 H, $^3J_{H7,H6}$ = 7.84 Hz, $^4J_{H7,F9}$ = 2.35 Hz, H-7), 5.522 (m, H-8), 4.050 (t, J = 6.32, H-1), 2.306 (brs, H-6), 2.025 (s, acetate), 1.61 (m, H-2), 1.30 (brs, H-3 and H-5); [^{19}F]NMR, no significant difference from Pfb-Z9–14:OH.

Electrophysiological Techniques

Insects. Adult *H. zea* and *T. ni* were obtained from a colony maintained by the Insect Attractants, Behavior, and Basic Biology Research Laboratory, Gainesville, Florida. Adult *D. grandiosella* males were obtained from a colony maintained by the Crop Science Laboratory, Mississippi State, Mississippi.

H. zea and T. ni. Details of the stimulus delivery device and procedures are described by Grant et al. (1989). The wings and legs of intact specimens were secured intact in a hollowed-out depression of a small Plexiglas plate with molten paraffin–beeswax. Basal and distal ends of the antenna were immobilized between strips of double-stick Scotch tape so that several distal flagellomeres (antennal segments) were accessible to the electrodes and stimuli.

Action potentials (spikes) were recorded by inserting uninsulated sharpened tungsten electrodes at the base of sensilla trichodea located over the lateroventral region of the antenna. The electrical responses were amplified by an AC-coupled Grass P-15D preamplifier and band-pass filtered (3–300 Hz). The impulse activity was monitored on a storage oscilloscope, and the signal was further amplified and led to a Digital PDP 11/23 computer. The digitized spike trains were stored and analyzed according to Mankin et al. (1987). The number of spikes per second was obtained by subtracting the number of spikes in the prestimulus interval from the number in the stimulus interval and dividing by the stimulus duration (3 sec).

The stimuli were delivered from a glass delivery device that maintained a constant flow of synthetic compressed air (Linde, Inc.) purified by passage first

through silica gel, then activated charcoal, and finally through silica gel. The constant airflow isolated the antenna from room air and carried the stimulus from the dispenser. The delivery device was rinsed with acetone and aerated following all stimuli at concentrations that produced a residual sufficient to elicit a response. The output of the stimulus delivery device was converted to concentration based on calibrations in Mayer et al. (1987). Vapor concentrations of the perfluoroalkyl analogs and nonfluorinated stimuli were essentially identical at the same dosage level (see calibration data below).

D. grandiosella. Electroantennogram (EAG) techniques were adapted from earlier methods (Schneider, 1957) and described elsewhere (Dickens, 1979; Hedin et al., 1986). Briefly, two glass microcapillary electrodes filled with physiological saline (Oakley and Schafer, 1978) were inserted into the antenna of an intact animal; the ground electrode was placed into the fourth or fifth antennal flagellomere, and the recording electrode was inserted into the terminal or penultimate antennal segment. Electrical contact was provided by a Ag–AgCl wire placed into the shank of each capillary. Electrical activity was amplified 10× by a Grass P-16 DC preamplifier. EAG waveforms were visualized on a Tektronix 5111 analog storage oscilloscope and recorded using a stripchart recorder. Stimulus duration was 1 sec with an airflow of 1 l/min. Three minutes were allowed between stimuli at lower dosages; 5–7 min were allowed at dosages greater than 1 mg. These times were adequate for complete recovery of the EAG.

The stimuli, Z11–16 : Al, Z9–16 : Al, Pfb-Z11–16 : Al, and Pfb-Z9–16 : Al, were delivered from the lowest to the highest dose. Filter papers (8 mm × 18 mm) were treated with 1-μl aliquots of serial dilutions and were inserted into glass cartridges (80 mm × 5 mm ID) that were oriented toward the preparation from a distance of 1 cm. Responses of three *D. grandiosella* males were recorded for each dose of each pheromone and pheromonal analog. 1-Hexanol (100 μg; 10 μl of 10 μg/μl dissolved in pentane) served as a standard to normalize responses, to ensure viability and constancy of the preparation, and to allow relative comparisons with previously recorded species (Dickens, 1984). Stimulation with the standard both preceded and followed each serial dilution level. No responses were obtained to a solvent control. EAGs were expressed as a percentage of the mean of the two nearest responses to the standard (Dickens, 1981). The threshold response was considered to be the dosage at which the standard error of the mean was not equal to or less than zero (Dickens, 1984).

Single-cell recording techniques are described in detail elsewhere (Dickens, 1979; Dickens and Mori, 1988) and were modified from Boeckh (1962). In brief, microelectrodes used for recordings were constructed from 50.8-μm-diameter tungsten wire electrolytically sharpened to a tip of 1–2 μm. The recording electrode was positioned under optical control (150–200×) by a Leitz micromanipulator near the base of a single sensillum trichodeum. The ground

electrode was implanted in a distal antennal segment. Action potentials were amplified by a Grass P-15 preamplifier and displayed on a Tektronix 5223 digitizing oscilloscope. The signal was recorded on a Teac R51-D data recorder and displayed on a Tektronix 5111 analog oscilloscope during storage on the hard disk of a Dell Corporation 286 microcomputer. Trains of action potentials were counted visually from the oscilloscope screen or analyzed with the aid of the microcomputer utilizing SAPID (Spike Analyses Programs for Insect Data) from Tasteful Software Laboratory, Department of Entomology, University of Alberta, Edmonton, Alberta. The stimulus delivery system was the same as that described for single cell stimulations; stimulus duration was 0.5 sec.

Quantification of Analogs by Gas Chromatography

Detector Response Factors. For Z9–14 : Ac, Pfb-Z9–14 : Ac, and *n*-hexadecane, doses of 1–50 ng were injected into Varian 3700 gas chromatograph equipped with a capillary column (DB-5 0.263 mm ID × 30 m). A plot of the area response (by flame ionization detection) to these compounds vs. the amount injected was linear over this range. The relative area responses are: *n*-hexadecane (1.00), Z9–14 : Ac (0.91), and Pfb-Z9–14 : Ac (0.20). These tests were crucial in calibrating the GC for volatilization assays.

Volatilization of Analog during Preparation of Delivery System. The dispenser device used was that described by Mayer et al. (1984). The Z9–14 : Ac or Pfb-Z9–14 : Ac diluted in 0.5 ml of hexane was pipetted into the assembly. The dispenser was rotated under aeration at 200 ml/min for 30 sec to coat the inside uniformly. Then the dispenser was washed with hexane (2 × 1 ml). The recoveries of both Z9–14 : Ac and Pfb-Z9–14 : Ac at 10-, 50-, and 100-μg dosages were over 95% as determined by GC. This test was used to confirm that there were no gross differences in physical properties for the fluorinated and nonfluorinated compounds that would affect release from the dispenser device.

RESULTS

Synthesis of Polyfluoroalkyl Pheromone Analogs

Starting from commercially available 3-octyn-1-ol (I), 3-decyn-1-ol (II), and 7-dodecyn-1-ol (III), the perfluoroalkyl pheromone analogs XV, XVI, XVII, and XVIII could be synthesized in four steps in 40–45% overall yield (Figure 1). For simplicity, we employ the prefix "Pfb" to indicate the perfluorobutyl modification and "Pfh" to indicate the perfluorohexyl modification.

The synthesis of Pfb-Z9–14 : Al (XV) is described as a representative example. 9-Decyn-1-ol (V) was obtained from 3-decyn-1-ol (II) in 85% yield according to the zipper reaction as described by Abrams and Shaw (1987). The

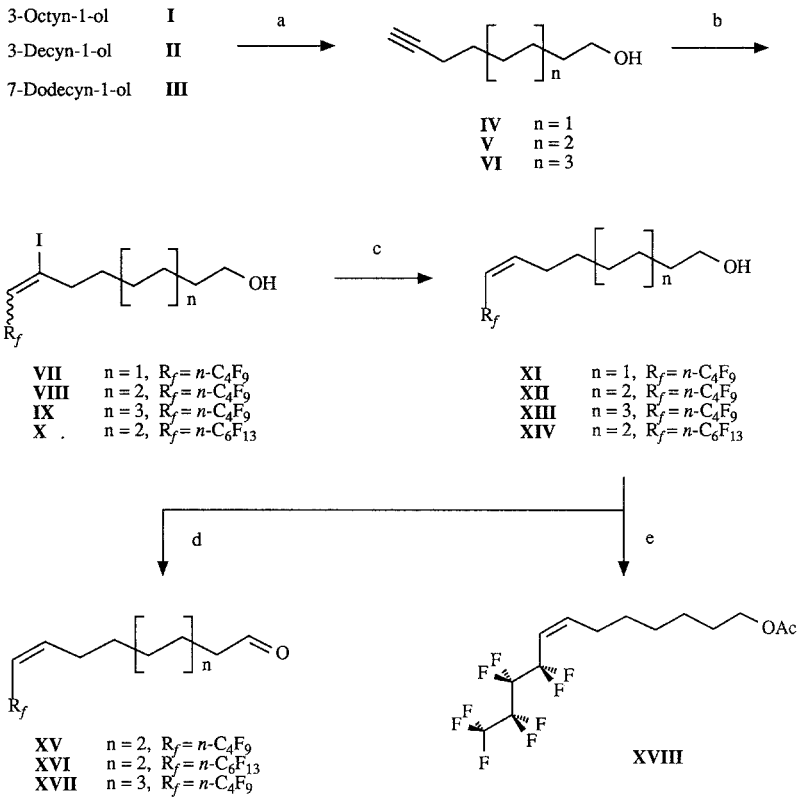

FIG. 1. Synthesis of perfluoroalkyl pheromones. Reagents and conditions: (a) NaH, 1,3-diaminopropane, 80°C, 2 hr, 80–85% yield; (b) n-C_4F_9I or n-$C_6F_{13}I$, azobisisobutyronitrile, 80°C, 18 hr, 80–85%; (c) 1. SiO_2 separation, 2. n-BuLi, THF, −78°C, 0.5 hr, 3. MeOH, −78°C, 75–80%; (d) N-methylmorpholine N-oxide, tetrapropylammonium perruthenate, 4 Å molecular sieves, CH_2Cl_2, 14 min, 85–90%; (e) acetic anhydride, 4-dimethylaminopyridine, pyridine, 45°C, 3 hr, 94%.

ω-alkynol was heated with 1.5 equivalents of perfluorobutyl iodide and catalytic amount of azobis(isobutyronitrile) (AIBN) in a sealed heavy-wall glass tube for 18 hr under nitrogen atmosphere (Fuchikama and Ojima, 1984; Ishihara et al., 1986, Matsubara et al., 1987). The crude alkenyl iodide was isolated as a mixture of E and Z isomers with a ratio of 8 : 7 (Table 1). The E and Z perfluorobutyl vinyl iodides VIII could be separated by flash column chromatography; fractions with over 98% isomeric purity (GC) were combined and concentrated for subsequent reactions. It is important to separate the alkene isomers at this stage, as discussed below.

Deiodination of the (E)-alkenyl iodide VIII with n-butyllithium at −78°C

TABLE 1. KOVÀTS INDICES (GAS CHROMATOGRAPHY RETENTION INDICES)

	X = OH	X = Al	X = Ac
Perfluoroalkyl analogs			
Pfb-Z11-16:X	1744	1558	1744
Pfb-Z9-14:X	1427	1366	1558
Pfb-Z7-12:X	1250	1185	1363
Pfh-Z9-16:X			1646
Natural pheromone components			
Z11-16:X		1891	1982
Z9-14:X		1672	1776
Z7-12:X			1577

followed by quenching with methanol at that temperature afforded the pure *Z* isomer XII with retention of geometry. (Note that the priority rules give iodide a higher priority than alkyl; nonetheless, the alkenyl chain is *cis* in both compounds.) It is important to keep the concentration of the iodide below 0.1 M to provide good yield and a clean reaction, since the solubility of the dianion was a determining factor in this lithium–halogen exchange reaction. Oxidation (TPAP, NMO, CH_2Cl_2) or acetylation (Ac_2O, DMAP, CH_2Cl_2) of the perfluorobutyl alkenol provided the Pfb-Z9-14:Al (XV) or Pfb-Z7-12:Ac (XVIII) [from Pfb-Z7-12:OH (XIV)] in very good yields. It is worth noting that the separation of *E* and *Z* isomers by flash column chromatography was only possible on the perfluorobutyl alkenyl iodides. The *E* and *Z* isomers of all subsequent perfluoroalkyl analogs were inseparable by silica gel chromatography, even using argentation conditions known to separate the corresponding nonfluorinated compounds.

The perfluoroalkyl pheromone analogs are significantly more volatile than their parent compounds, as shown by the lower values for the Kovàts retention indices (Kovàts, 1958) (Table 1). Thus, even though the molecular weights of the perfluorobutyl compounds are 128 mass units higher, the loss of intermolecular hydrophobic interactions renders the molecules less cohesive in the condensed phase. For the aldehydes, adding nine fluorines is the equivalent of shortening the chain by three methylene units!

The concentration of the perfluoro analogs delivered to the antenna is essentially the same as that of the natural pheromone stimulus. Quantification by GC can be deceptive, because the molar response to highly fluorinated molecules is drastically reduced because of the large portions of nonflammable $-CF_2-$linkages. Thus, detector response curves were generated for Pfb-Z9-14:Ac, *n*-hexadecane, and Z9-14:Ac from 1 ng to 50 ng/injection. Relative

to *n*-hexadecane (1.00), these factors were 0.91 for Z9–14:Ac and 0.20 for Pfb-Z9–14:Ac. Next, we determined that >95% of both Pfb-Z9–14:Ac and Z7–14:Ac remained in the delivery device after evaporation of 0.5 ml of hexane solutions containing 10, 50, and 100 μg of each compound.

Electrophysiological Recordings

H. zea. Recordings from neurons within the long, sexually dimorphic sensilla on the proximal 40 or so antennal flagellomeres demonstrated that a 200-μg dose of Pfb-Z11–16:Al elicits about the same number of spikes as a 0.02-μg dose of Z11–16:Al (Figure 2).

T. ni. Three different receptor neurons contained within two different types of sensilla responded to fluorinated analogs of their most effective pheromone stimuli. The HS sensillum has two highly sensitive and selective receptor neurons, one that responds to Z7–12:Ac [HS(a)] and another that responds to Z7–12:OH [HS(b)] (O'Connell et al., 1983). The perfluorinated analogs of each of these compounds elicited a response from the appropriate neuron at a dose of about 10 μg, compared with thresholds of about 0.02 μg of the native compounds (Figure 3). The difference in sensitivity between the perfluorinated analogs and the natural components, consequently, was about the same as the receptor neurons of *H. zea*.

Another highly selective and sensitive receptor neuron is found within a different sensillum on the antenna of the cabbage looper that responds to Z9–14:Ac (Mayer, unpublished). Both Pfb-Z9–14:Ac and Z9–14:Ac elicited a

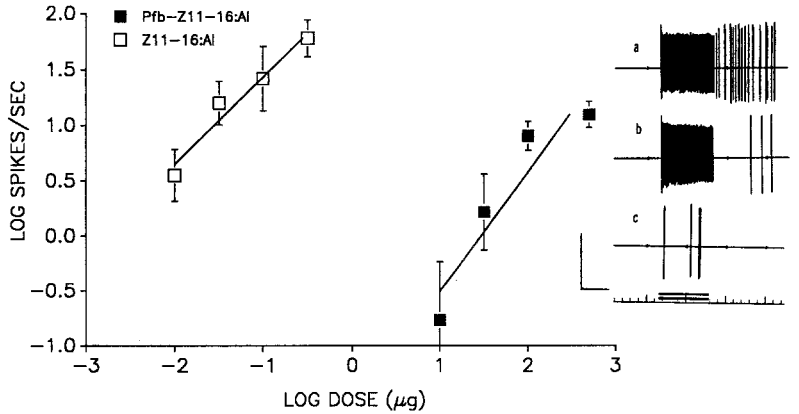

FIG. 2. Averaged responses (±SEM) of six *H. zea* olfactory receptor neurons to Z11–16:Al standards (open squares) and Pfb-Z11–16:Al (closed squares). Inset: response to (a) 1 μg Z11–16:Al; (b) 250 μg Pfb-Z11–16:Al; and (c) 10 μg of Pfb-Z11–16:Al. Calibration: vertical bar = 1 mV; horizontal bar = 1.5 sec.

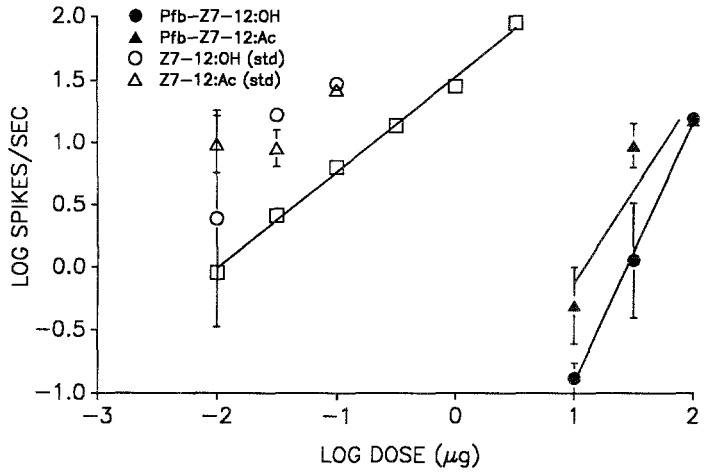

FIG. 3. Averaged responses (±SEM) of five *T. ni* HS(a) neurons to Pfb-Z7-12:Ac (closed triangles) and Z7-12:Ac (open triangles). Responses of the HS(b) olfactory receptor neurons within the same sensilla to Pfb-Z7-12:OH (closed circles) and Z7-12:OH (open circles). A standard response curve from a larger sample of HS(a) neurons is provided for reference (open squares) (Mayer, unpublished).

response from this neuron (Figure 4). The response elicited by a 32-μg dose of the perfluorinated analog was equivalent to a dose of about 0.01 μg of Z9-14:Ac, a difference of about four orders of magnitude.

D. grandiosella. Male antennae responded to serial dilutions of Z9-16:Al

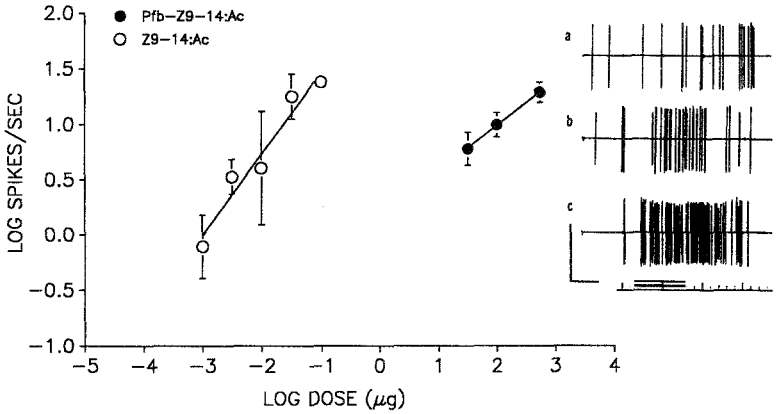

FIG. 4. Averaged responses (±SEM) of six *T. ni* olfactory receptor neurons to Z9-14:Ac (open circles) and Pfb-Z9-14:Ac (closed circles). Inset: responses to (a) 32 μg (b) 100 μg, and (c) 500 μg doses of Pfb-Z9-14:Ac. Calibration: vertical bar = 1 mV; horizontal bar = 1 sec.

and Z11-16:Al, showing thresholds at doses of <0.001 μg with saturation occurring at 1 μg for both compounds (Figure 5A and B). The EAG threshold for both Pfh-Z9-16:Al or Pfb-Z11-16:Al appears to be at doses of approximately 0.01-1 μg. Thus, the thresholds for the perfluoroalkyl analogs are about 100- to 1000-fold higher than the actual pheromone. Above threshold, the slope of the dose-response curve for each Pfb analog is similar to that for the native pheromone components.

Comparison of action potentials recorded from five sensilla trichodea on

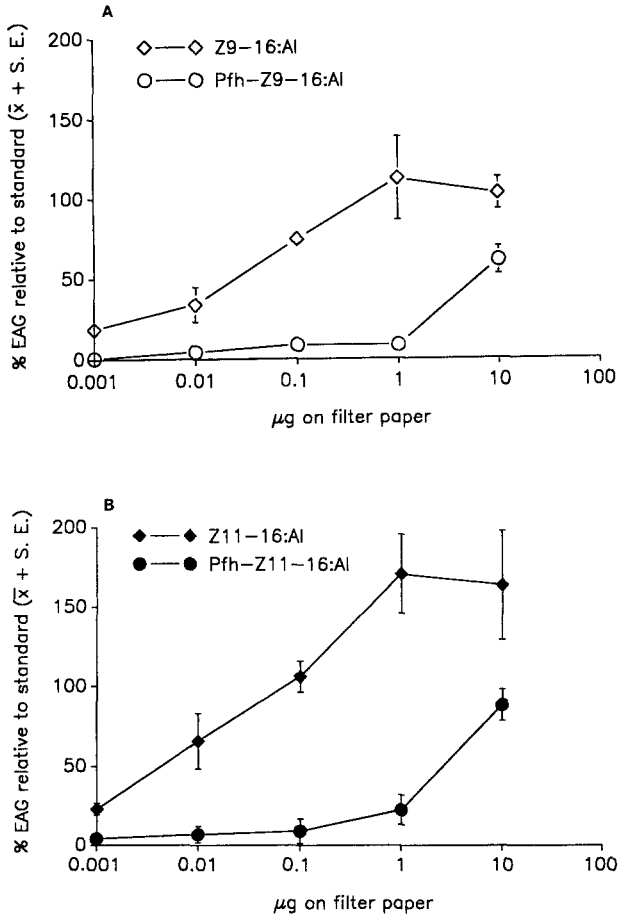

FIG. 5. Dose-response profiles for EAG recordings from *Diatraea grandiosella* male antennae. (A) Relative responses (±SEM) to Z9-16:Al (open diamonds) and Pfh-Z9-16:Al (open circles); (B) relative responses (±SEM) to Z11-16:Al (closed diamonds) and Pfb-Z11-16:Al (closed circles).

the male antenna revealed three receptor neurons responsive to Z9–16 : Al and three neurons responsive to Z11–16 : Al. Two of the neurons responsive to each pheromone component were also activated at a low level by the perfluorobutyl analogs. For example, 1-μg doses of a Z9–16 : Al elicited 80 spikes/initial 400 msec, while a dose of 10 μg of Pfh-Z9–16 : Al elicited 15 spikes/initial 400 msec (spontaneous activity = 3 spikes/400 msec). These results are consistent with the EAG results and indicate the ability of these analogs to stimulate their respective pheromone receptor cell neurons.

DISCUSSION

Volatile signals involved in chemical communication in insects are known to have narrowly defined criteria of molecular structure and physical properties. Among the key molecular properties are chain length and branching, type and location of functionality, degree of unsaturation, alkene geometry, and chirality. The macroscopic properties that affect both emission and reception of a chemical signal include volatility, lipophilicity, dipole moment, and chemical stability. On the molecular level, the introduction of perfluoroalkyl chains into insect pheromones dramatically increases chain bulkiness and reduces chain flexibility and hydrophobicity (Filler and Kobayashi, 1982). At the macroscopic level, the polyfluoroalkyl compounds are more volatile, less lipophilic, have larger dipole moments, and are more stable. Recently, this general class of semifluorinated hydrocarbons was described as "primitive surfactants" (Turberg and Brady, 1988), based on their unusual physical properties and the dual polar–nonpolar nature imparted by perfluorination of one end of a hydrocarbon chain. Stated simply, perfluoroalkyl groups have unique properties that reduce their solubility in both aqueous (hydrophilic) and lipid (hydrophobic) environments.

Bestmann and coworkers (Bestmann et al., 1987; Bestmann and Vostrowsky, 1982; Bestmann, 1986) and others over the last two decades have provided evidence from extensive EAG analyses that demonstrates the importance of alkyl chain length, alkene geometry, alkene position, branching, and functionality in determining the electrophysiological activity of insect pheromones and pheromone analogs. The existence of a chiral recognition site on a macromolecule also was inferred through the demonstration that chiral analogs of an achiral pheromone were perceived differently (Chapman et al., 1978). Energetic profiles of receptor site interactions were discussed in a theoretical sense by Kafka (1976) and by Kafka and Neuwirth (1975), and early efforts to correlate moth sex pheromone activities with molecular conformations were described by Kikuchi (1975). However, major computational advances have been introduced recently by chemical ecologists in the Lund group. The con-

formational energy of pheromone analogs calculated by molecular mechanics can now be correlated with electrophysiological potency (Liljefors et al., 1985, 1987), thus providing experimental evidence supporting a specific macromolecular binding site with spatial recognition for a relatively small set of steric and electronic features.

The responses of pheromone-sensitive receptor neurons in two species of noctuid moths and one pyralid moth to fluorinated pheromone analogs provide a new set of data in support of the importance of the hydrophobicity of the terminal alkyl group in pheromone–receptor protein interactions. In each of the three species examined, the pheromone receptor neurons that are specialized to detect particular pheromone components responded to the homologous perfluorinated molecules, although at elevated concentrations. *H. zea* sensilla that contain receptor neuron(s) responding to Z11–16:Al also were stimulated by the perfluoro analogs. Some other neurons within these same sensilla that responded to Z11–16:Al failed to respond to the Pfb analogs, however. Moreover, the neuron in the HS sensillum of *T. ni* that responds to Z7–12:OH did not respond to the perfluorinated analog of Z7–12:Ac; neither did the neuron recognizing Z7–12:Ac respond to Pfb-Z7–12:OH. Thus, the neuronal responses are consistent with the notion that although perfluorination changes the polarity, hydrophobicity, and conformational rigidity of the pheromone molecule, it does not change it sufficiently to completely alter the overall shape or the dependence on alkene position, alkene geometry, or terminal functionality.

Another feature of the neuronal response to these fluorinated analogs is common to *H. zea* and *T. ni* and, by inference, to *D. grandiosella*. Usually, following the response to high concentrations of the native pheromone components of *T. ni*, there is an elevated poststimulus discharge frequency (Grant et al., 1989). Responses to perfluorinated compounds in both *H. zea* and *T. ni* resulted in a reduced poststimulus discharge by most neurons at any concentration assayed (Figure 2). We interpret this phenomenon in two ways. First, the high concentrations of these compounds required to elicit an increase in spike frequency creates significant demands on their purity. For example, a 0.2% impurity of Z11–16:Al in a dose of over 500 μg means that 1 μg would be in the sample. This amount is sufficient to elicit a significant response by the receptor neurons (Figure 2, inset a). Thus, the lack of a poststimulus discharge (Figure 2, inset b) suggests that the response to the perfluorinated compound is not due to a Z11–16:Al contaminant, which would also be unexpected based on the synthetic scheme.

Second, the diminished poststimulus discharge suggests that although the less hydrophobic perfluorobutyl analogs of Z11–16:Al can stimulate the receptor that recognizes their shape, functionality, and chain length, the reduction in dispersive interaction results in their more rapid dissociation from the receptor. This lack of an increased poststimulus spike frequency also is observed in

response to unphysiologically high concentrations of pheromone components that are poor stimuli of a receptor neuron (Mayer, unpublished). Thus, on the basis that fluorinated analogs act as ligands with poor affinity for hydrophobic regions of pheromone receptors, we have categorized this class of compounds as "nonstick" pheromone analogs. We further speculate that the receptor site may react similarly to other inappropriate pheromonal stimuli at unphysiologically high concentrations.

Two decades of work by Kaissling (1986) led to the hypothesis that long recoveries are due to slow inactivation or slow removal of the stimulant. If this were true for the perfluoroalkyl analogs, then increased removal or inactivation would be required. We prefer the alternative kinetic explanation, i.e., that reduced receptor affinity requires a higher concentration of stimulant to achieve the same level of receptor activation and subsequent signal transduction.

Two very recent precedents that corroborate our observations can be seen in the responses of a moth and a mosquito to fluorinated pheromone analogs. First, the western spruce budworm, *Choristoneura occidentalis*, shows a 14-fold shorter recovery period in EAG responses to the terminally fluorinated analog 14-F-*E*11–14 : Al relative to the recovery period for responses to *E*11–14 : Al (McLean et al., 1989). These researchers suggested increased degradation and transport as the primary explanation for the shortened recovery period. However, both their results and ours seem to provide stronger evidence for the primary effect being the alteration of the hydrophobicity of the terminal methyl group and thus reduction in receptor affinity.

Second, the Pickett group in Rothamsted (Dawson et al., 1990) has independently discovered biological activity in perfluorinated pheromone analogs. In their example, a C_8F_{17} group was substituted for a terminal octyl group in an analog of (−)-(5*R*,6*S*)-6-acetoxyhexadecanolide, the oviposition attractant of the mosquito *Culex quinquefasciatus*. This analog was a potent oviposition stimulant in field tests.

Current biochemical evidence on the molecular mechanism of olfaction in vertebrates (Pace and Lancet, 1987; Snyder et al., 1988) and insects (Vogt, 1987) suggests that pheromones associate with pheromone-specific soluble carrier proteins in the olfactory hair lumen and with membrane-associated receptor proteins on the dendritic surface. For example, in the moth *Antheraea polyphemus*, a male-specific sensory hair membrane protein (69 kDa) and a soluble pheromone binding protein (15 kDa) have been selectively photoaffinity labeled using a physiologically active diazoacetate analog of the pheromone (*E*,*Z*)-6,11-hexadecadienyl acetate (Vogt et al., 1988; Ganjian et al., 1978). That a pheromone analog of similar size and shape, but dramatically different lipid solubility, exhibits pheromonal activity at a reduced level, is most consistent with a key role for a macromolecular receptor-pheromone complex in olfactory transduction.

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CONVENIENT SYNTHESIS OF MOSQUITO OVIPOSITION PHEROMONE AND A HIGHLY FLUORINATED ANALOG RETAINING BIOLOGICAL ACTIVITY

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Abstract—A simple three-step synthesis is described for 6-acetoxy-5-hexadecanolide, the oviposition pheromone of the mosquito *Culex quinquefasciatus* Say and others in that genus. An aldol condensation between 1-trimethylsilyloxycyclopent-1-ene and undecanal, followed by Baeyer-Villiger ring expansion and acetylation, gave the required compound as a 1:1 mixture of diastereoisomers in high overall yield (>80%). This synthetic approach is readily adapted for synthesis of analogs. The heptadecafluoro compound, in which the *n*-octyl group is replaced by perfluorooctyl, retained high biological activity.

Key Words—Mosquito, *Culex quinquefasciatus*, Diptera, Culicidae, oviposition, attractant, pheromone, synthesis, acetoxyhexadecanolide, perfluoroalkyl, pheromone analog.

INTRODUCTION

Mosquitoes in the genus *Culex* such as *Cx. quinquefasciatus* Say (= *Cx. pipiens fatigans* Wied.) release a pheromone from droplets that form on the apex of eggs as they mature. The pheromone attracts gravid females, which then ovi-

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posit in the region of the original laying (Bruno and Laurence, 1979). This pheromone comprises (−)-(5*R*, 6*S*)-6-acetoxy-5-hexadecanolide, Ia (Figure 1) (Laurence and Pickett, 1982; Laurence et al., 1985). Although the pheromone exists as the stereoisomer Ia, the other three isomers are inactive and do not inhibit the activity of the natural isomer (Laurence et al., 1985; Laurence and Pickett, 1985; Hwang et al., 1987). Pheromone from the new large-scale synthetic route described here (E.P. 00078641) has been used successfully in field trials in Kenya: mosquitoes were attracted to lay eggs at chosen sites, and the larvae were destroyed by incorporating into the pheromone formulation a juvenile hormone type larvicide, which presents no hazard to man or to the environment (Otieno et al., 1988).

Shortening the alkyl chain R in this molecule, for example Ib (Figure 1), caused complete loss of pheromonal activity at the levels tested (Laurence and Pickett, 1985). However, by maintaining the length of R and replacing the methyl group R′ in the acetoxy function with trifluoromethyl, the resultant analog Ic (Figure 1), although readily hydrolyzed in water, retained very high activity (Briggs et al., 1986). At first sight, this result is surprising. However, the trifluoroacetoxy analog retains all the structural features of the parent pheromone. Although the electron-withdrawing properties of the trifluoromethyl group would increase the electrophilicity of the ester carbonyl carbon, interactions between the oxygenated part of the molecule and the pheromone receptor would not be qualitatively different from interactions between the natural pheromone and the receptor. In contrast, fluorine substitution for hydrogen in the

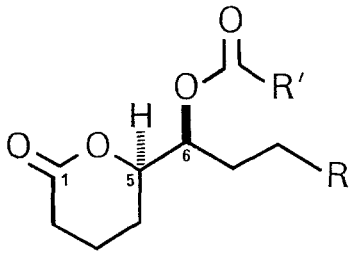

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FIG. 1. Structures of the mosquito oviposition pheromone and analogs: Ia, R = $(CH_2)_7CH_3$, R′ = CH_3; Ib, R = $(CH_2)_3CH_3$, R′ = CH_3; Ic, R = $(CH_2)_7CH_3$, R′ = CF_3; Id; R = $(CF_2)_7CF_3$, R′ = CH_3; Ie, R = $(CF_2)_7CF_3$, R′ = CF_3. The isomeric composition is described in the text.

alkyl chain R was expected to have a dramatic effect on pheromonal activity, because interactions between the alkyl chain and the receptor would be substantially weakened by the various physical changes resulting from fluorine replacement of hydrogen, such as the increased rigidity of the carbon chain (Banks and Tatlow, 1986). Indeed, lipophilic interaction would be essentially eliminated when the alkyl chain was perfluorinated as interaction with a hydrocarbon receptor site would be substantially disrupted (Prestwich et al., 1990). It was therefore of interest to devise a synthetic route to such compounds in order to investigate their interaction with *Culex* spp. mosquitoes. Here we describe synthesis and bioassay of the heptadecafluoro compound Id (Figure 1), i.e., the perfluorooctyl analog, in which fluorine replaces all hydrogens in R except for the two methylene groups adjacent to the acetoxy function. These were left unchanged, in order that the electron densities in the oxygenated part of the molecule would remain largely unaffected by the fluorine substitution.

Many syntheses of the mosquito pheromone have been described. Key synthetic steps have included bishydroxylation of (*Z*)-5-hexadecenoic acid ester (Laurence and Pickett, 1982) and epoxidation of (*E*)-5-hexadecenoic acid (Ochiai et al., 1985) for racemic material. The Sharpless enantioselective epoxidation of 1-tridecen-3-ol (Mori and Otsuka, 1983; Barua and Schmidt, 1986) and 2-tridecen-1-ol (Lin et al., 1985) also have featured prominently. Two other groups have used the protected chiral 2-hydroxydodecanal in reaction with 4-pentenylmagnesium bromide to achieve separable diastereoisomers (Fuganti et al., 1982; Ko and Eliel, 1986) with further elaboration to the target molecule. (*S*)-2-Cyclohexen-1-ol has been used to provide two diastereomeric epoxides, which have been regioselectively alkylated with decyllithium to give both *erythro* enantiomers (Sato et al., 1984). All four stereoisomers have been prepared using isopropylidine-(*R*)- or -(*S*)-glyceraldehyde as a chiral synthon (Machiya et al., 1985). However, in order to provide sufficient material for field experiments, a synthesis with few and high yielding steps was required, and a simple three-step route was proposed. This utilized an aldol condensation between cyclopentanone and undecanal to furnish the correctly substituted cyclopentanone, which was expected to undergo the Baeyer-Villiger ring expansion as required (Figure 2). Acetylation then would achieve the product in a three-step synthesis. Analogs/isosteres would be readily prepared by adapting this scheme.

During the course of this work, another simple three-step synthesis was published in which acrolien dimer was reacted with decylmagnesium bromide, the product acetylated, and the cyclic enol ether converted to the lactone (Jefford et al., 1986). This scheme showed the potential for good diastereoselective control. Also, a referee brought to our attention a paper given at the Huang Minlon symposium in November 1989, now published (Wang et al., 1990), describing a different synthesis which employed a Baeyer-Villiger strategy.

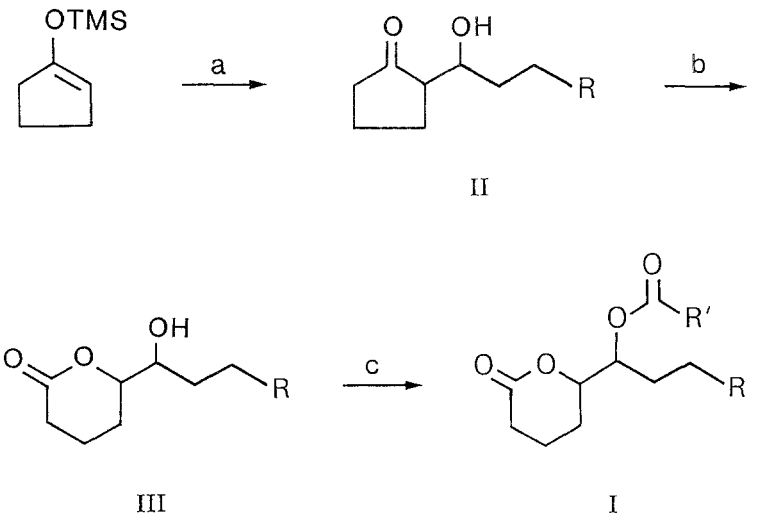

FIG. 2. Synthetic route to mosquito oviposition pheromone and analogs: (a) RCH_2CH_2CHO, $TiCl_4$, (b) *m*-chloroperbenzoic acid, KF, (c) $(R'CO)_2O$, Py (see Figure 1 for R'). I see Figure 1, IIa, IIIa: R = $(CH_2)_7CH_3$; IId, IIId: R = $(CF_2)_7CF_3$.

METHODS AND MATERIALS

General Methods

Gas chromatography (GC) was performed with a Pye Unicam 204 gas chromatograph with a flame ionization detector and fitted with a 5 m × 0.3 mm ID OV-101 bonded fused silica capillary column operated isothermally at 180°C. Results are expressed as: retention time (minutes), composition (percent), Kovats index. Mass spectrometry (MS) employed a VG Analytical Ltd. 70–250 mass spectrometer equipped with thermospray–plasmaspray source with the sample delivered in 80:20 methanol–water with the source at 220°C. Accurate mass measurements were performed in the electron impact (EI) mode on probe-tip samples with a source temperature of 250°C using a software peak-matching technique. The EI spectra obtained from probe samples showed predominantly M+1 peaks in the region of the molecular ion, as reported by other workers (Lin et al., 1985). Satisfactory proton nuclear magnetic resonance ([^{1}H]NMR) spectra (400 MHz) were obtained for all compounds. However, carbon nuclear magnetic resonance spectra ([^{13}C]NMR) were most valuable for characterizing these compounds, and the data are reported in full (Table 1) using a JEOL GNX 400 spectrometer at 100 MHz in $CDCl_3$ solution with tetramethylsilane (δ = 0.0) as internal standard. Dichloromethane was refluxed and

TABLE 1. [^{13}C]NMR ASSIGNMENTS (ppm) OF MOSQUITO OVIPOSITION PHEROMONE, ANALOGS, AND SYNTHETIC INTERMEDIATES

Compound	Carbon number[a]								
	1	2	3	4	5	6	7	8	Others
IIa									
erythro	221.6	39.2	20.7	22.7	54.5	69.6	35.1	26.1	
threo	223.4	38.5	20.6	24.9	53.9	72.2	35.2	26.8	
IId									
erythro	221.6	38.9	20.5	23.2	54.9	68.7	25.3[c]	28.0[d]	
threo	225.0	38.3	20.5	26.6	53.7	71.2	25.8[c]	26.7[d]	
IIIa									
erythro	171.7	29.7	18.5	21.4	83.6	72.4	31.9	25.9	
threo	171.9	29.7	18.5	24.2	83.3	73.3	32.7	25.6	
IIId									
erythro	171.4	29.7	18.3	21.4	83.1	71.4	23.6[c]	27.8[d]	
threo	171.0	29.6	18.3	24.1	83.3	72.5	22.5[c]	27.3[d]	
Ia									
erythro	170.7/170.3[b]	29.7	18.2	23.5	80.5	74.3	29.7	25.2	20.9
threo	170.6/170.8[b]	29.7	18.4	24.1	79.8	73.9	29.9	25.4	20.9
Id									
erythro	{170.5, 170.4}[b]	29.6	18.3	23.9	80.0	73.0	21.3[c]	27.1[d]	20.8
threo	{170.4, 170.3}	29.6	18.3	23.9	79.7	72.7	20.7[c]	27.3[d]	20.9
Ic[b]	172.3	29.6	18.1	22.6	80.4	78.8	29.6	24.9	114.6 (2q, J = 286)
	172.4	29.6	18.2	23.9	79.7	78.7	29.6	25.1	157.0 (q, J = 42)
									157.1 (q, J = 42)
Ie									
erythro	169.9	29.5	18.1	23.5	79.2	77.2	20.9[c]	26.8[d]	114.4 (2q, J = 286)
threo	170.0	29.6	18.3	23.8	78.9	76.9	21.1[c]	26.9[d]	157.0 (q, J = 43)
									157.1 (q, J = 43)

[a] For convenience the numbering in this table is based on the acyclic form of the lactone, and this is also used correspondingly in the aldol product IIa, d.
[b] Not assigned.
[c] Broadened triplet J = 3 for R = $-(CH_2)_7CH_3$ 29.7(5), 31.8, 22.7, 14.1.
[d] Triplet J = 22 for R = $(CF_2)_7CF_3$ weak signals split δ = 108 − 120.

distilled from phosphorus pentoxide. Titanium tetrachloride was distilled under nitrogen and stored in dry dichloromethane solution.

Synthesis

2-(1-Hydroxyundec-1-yl)cyclopentanone, IIA. Titanium tetrachloride (9.5 g, 0.05 mol) in dichloromethane (40 ml) was stirred at −78°C under nitrogen and treated dropwise with a mixture of 1-trimethylsilyloxy-1-cyclopentene (7.87 g, 0.05 mol), (bp 60–61°C, 25 torr, from cyclopentanone, chlorotrimethylsilane, potassium iodide, and triethylamine in dimethylformamide) and freshly distilled 1-undecanal (8.5 g, 0.05 mol) over 0.25 hr. Stirring was continued for a further 0.5 hr, water (50 ml) added, and the mixture partitioned by addition of hexane (100 ml) and water (100 ml). The organic layer was washed with water (3 × 50 ml) and with saturated sodium hydrogen carbonate (70 ml). After evaporation, the residue (13.3 g) was analyzed by [^{13}C]NMR spectroscopy and immediately submitted to the next step.

Separation of the diastereoisomers may be achieved on silica eluting with increasing amounts of diethyl ether in hexane. Conversion of these to the lactones (see below) and comparison with an authentic sample from earlier work (Laurence and Pickett, 1982) allowed assignment of the *erythro* product. The *threo* diastereomer was assigned by difference.

6-Hydroxy-5-hexadecanolide, IIIa. The crude hydroxyundecylcyclopentanone (13.3 g) in dichloromethane (100 ml) was treated with *m*-chloroperbenzoic acid (85%, 10 g, 0.05 mol) with water cooling. After 3.0 hr stirring, potassium fluoride (3.0 g, 0.05 mol) was added, and the resulting insoluble complexes were removed by filtration. The solid was washed with dichloromethane (100 ml) and the filtrate concentrated. The residue was dissolved in hexane (100 ml), and the precipitate that formed was removed (10.75 g) and further solid obtained by reducing the volume of the mother liquors (0.85 g). The solid (11.6 g, 86% overall yield from aldol and Baeyer-Villiger reactions) was analyzed by [^{13}C]NMR spectroscopy and GC.

GC *erythro* 3.60 (50%), 2198, *threo* 3.90 (50%), 2216.

6-Hydroxy-5-hexadecanolide, IIIa (via Lithium Enolate). Diisopropylamine (2.52 g, 0.025 mol) in dimethoxyethane (10 ml) at −15°C under N_2 was treated with butyllithium (9.5 M) in hexane (2.8 ml, 0.025 mol) and the mixture left for 0.5 hr. Then, at −78°C, cyclopentanone (2.1 g, 0.025 mol) was added followed, after 0.5 hr, by 1-undecanal (4.25 g, 0.025 mol) and the reaction left for a further 0.5 hr. Aqueous acetic acid was added to pH 5, followed by water (100 ml) and hexane (50 ml) and the mixture partitioned. The organic phase gave, after washing with water (2 × 50 ml), drying, and concentrating, a clear oil (6.0 g), which was analyzed by [^{13}C]NMR spectroscopy. Comparison with an authentic spectrum suggested that the aldol yield was approximately 65%,

of which 50% was *erythro* and 15% *threo*. This was confirmed by converting half the product (3.0 g) with *m*-chloroperbenzoic acid to the lactone, which was obtained from hexane as a solid product (0.9 g, 28%) comprising 1 : 1 *erythro–threo* isomers and recovering the pure *erythro* diastereomer from the mother liquors (1.05 g, 32.7%) as a colorless oil.

6-Acetoxy-5-hexadecanolide, Ia. The hydroxyhexadecanolide (5.0 g) was treated with acetic anhydride (10 g) and pyridine (10 ml) and left at room temperature overnight. Volatile material was removed under reduced pressure using added portions of toluene until constant weight (5.7 g, 100%) was achieved.

GC *erythro* 4.90 (50%), 2287, *threo* 5.20 (50%), 2303.

6-Trifluoroacetoxy-5-hexadecanolide, Ic. The hydroxyhexadecanolide (1.0 g, 3.7 mmol) and pyridine (1.05, 10.3 mmol) in diethyl ether (10 ml) were treated dropwise with trifluoroacetic anhydride (1.55 g, 7.4 mmol) with stirring and cold water cooling. The reaction was quenched with water (10 ml) and partitioned. The organic phase was washed with water (2 × 10 ml), dried, and concentrated to leave a colorless oil (1.25 g, 93%).

GC *erythro* 2.40 (50%), 2071, *threo* 2.65 (50%), 2102.

4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoroundecanal. Potassium 2,2,3,3-tetrahydroperfluoroundecanoate (5.3 g, 0.01 mol, Fluorochem) in thionyl chloride (10 ml) was stirred at room temperature for 2.0 hr with one drop of dimethylformamide and the reaction completed under reflux for 0.25 hr. Volatile material was removed under reduced pressure to leave the crude acid chloride (4.7 g).

The crude acid chloride (4.7 g,) in diethyl ether (50 ml) was added slowly to lithium aluminum hydride (0.5 g, 0.013 mol) in diethyl ether (20 ml) and left stirring at room temperature for 1.0 hr. Excess hydride was destroyed by addition of ethyl acetate and the complexes destroyed by addition of water (0.5 ml), aqueous sodium hydroxide (2 N, 0.5 ml), and water (1.5 ml). The resulting precipitate was removed by filtration. The solid was washed with diethyl ether and the filtrate concentrated (3.7 g, 79%) and analyzed by [^{13}C]NMR spectroscopy.

[^{13}C]NMR 24.0, 28.3 (t, $J_{\underline{C}H_2\text{-}CF_2}$ = 23), 61.7.

Pyridinium chlorochromate (2.0 g, 0.013 mol) in dichloromethane (25 ml) with the unpurified alcohol (3.7 g, 0.007 mol) was stirred for 3.0 hr. Diethyl ether (50 ml) was added, and the supernatant liquid was decanted from the insoluble material and passed through a short column of Florisil (10 g) to remove remaining inorganic material. More ether (20 ml) was passed through the column, the eluate concentrated, and the heptadecafluoroundecanal crystallized from hexane (2.5 g, mp 39–41°C, 67%).

[^{13}C]NMR 24.1 (t, $J_{\underline{C}H_2\text{-}CF_2}$ = 24), 35.0, 198.0.

2-(1-Hydroxy-4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoroundec-1-yl)cyclopentanone, IId. Titanium tetrachloride (0.6 g, 0.002 mol) in

dichloromethane (10 ml) at −78°C, under nitrogen, was treated dropwise with a solution of the heptadecafluoroundecanal (0.95 g, 0.002 mol) and 1-trimethylsiloxy-1-cyclopentene (0.315 g, 0.002 mol) in dichloromethane (10 ml). After 0.5 hr under these conditions water (20 ml) was added, and the organic layer was separated, washed with water (3 × 50 ml), with saturated sodium hydrogen carbonate solution (10 ml), dried, and concentrated leaving a yellow oil (1.2 g) that was not purified further.

9,9,10,10,11,11,12,12,13,13,14,14,15,15,16,16,16-Heptadecafluoro-6-hydroxy-5-hexadecanolide, IIId. The crude aldol product (1.2 g) was added to a solution of *m*-chloroperbenzoic acid (1.0 g) in dichloromethane (20 ml) and stirred for 3.0 hr at room temperature. Potassium fluoride (0.3 g) was added, and the solid was filtered off and washed with dichloromethane (50 ml). The filtrate was concentrated, and the solid residue (1.15 g) was triturated with hexane leaving a white solid (0.82 g, 71%, based on aldehyde).

MS (plasmaspray); MH^+ 577. $C_{16}H_{13}F_{17}O_3$ (FW) = 576. (EI); MH^+ 577.065 (obs.), 577.067 (calc. for $C_{16}H_{13}F_{17}O_3$).

6-Acetoxy-9,9,10,10,11,11,12,12,13,13,14,14,15,15,16,16,16-heptadecafluoro-5-hexadecanolide, Id. The Baeyer-Villiger product (0.1 g) was treated with acetic anhydride (0.1 g) and pyridine (0.1 g) and left overnight at room temperature. Volatile material was removed under reduced pressure leaving an oil (0.11 g).

GC *erythro* 1.05 (55%), 1824, *threo* 1.10 (45%), 1836. MS (plasmaspray); MH^+ 619. $C_{18}H_{15}F_{17}O_4$ (FW) = 618. (EI); MH^+ 619.073 (obs.), 619.077 (calc. for $C_{18}H_{15}F_{17}O_4$).

6-Trifluoroacetoxy-9,9,10,10,11,11,12,12,13,13,14,14,15,15,16,16,16-heptadecafluoro-5-hexadecanolide, Ie. The Baeyer-Villiger reaction product (0.1 g) was treated with trifluoroacetic anhydride (0.1 g) at room temperature for 1.0 hr and the volatile material removed under reduced pressure leaving a yellow oil (0.12 g).

MS (plasmaspray). No mass spectrum for the parent compound was found, although a MH^+ 577, corresponding to the heptadecafluorohydroxyhexadecanolide $C_{16}H_{13}F_{17}O_3$ (FW) = 576, was observed, suggesting that there had been decomposition in the aqueous solvent system.

Bioassay

The heptadecafluoro pheromone analog Id was tested using two strains of *Cx. quinquefasciatus*, from Lagos, Nigeria, and Colombo, Sri Lanka. The compound (0.3 μg in 6 μl hexane) was applied to a polystyrene disk placed treated side upwards floating in a dish of water. The numbers of egg rafts laid were

compared with those in a control dish where the disks had been treated with hexane only, as described previously (Laurence and Pickett, 1985).

RESULTS AND DISCUSSION

The nonstereoselective synthesis of the mosquito pheromone described here, using an aldol methodology (Mukaiyama et al., 1974), is very convenient because the subsequent Baeyer-Villiger reaction yields a 1 : 1 mixture of diastereomers, which are readily purified by crystallization. This obviates the need for chromatography since the final acetylation step is quantitative. However, the use of a lithium enolate of cyclopentanone in the aldol reaction results in a 50 : 15 *erythro–threo* ratio (65% yield). Baeyer-Villiger reaction on this product again permits isolation of a 1 : 1 *erythro–threo* mixture from hexane, leaving pure *erythro* product in the mother liquors. This diastereoselectivity makes the use of a chiral lithium amide an exciting possibility for achieving enantioselectivity.

In the bioassay employed for the Lagos strain of the mosquito, 74% of egg rafts were laid in the treated dish for compound Id (difference between numbers of egg rafts for treated and untreated, significant at $P < 0.05$) and for the Colombo strain, 69% ($P < 0.02$). The synthetic unfluorinated pheromone Ia generally gives scores of over 80% for the Lagos strain and of 60–68% for the Colombo; therefore the heptadecafluoro analog of the pheromone clearly retains high pheromonal activity. In contrast, the trifluoroacetoxy analog Ie of the heptadecafluoro compound, prepared by treating the hydroxy-δ-lactone with trifluoroacetic anhydride, was essentially inactive (Lagos strain, 56%, $P > 0.1$, and Colombo, 55% $P > 0.3$), probably because this compound rapidly hydrolyzes as noted in the plasmaspray MS experiment. The stability of 6-trifluoroacetoxy-5-hexadecanolide has also been examined. Exposure of a sample of this compound to the air for 35 days showed no remaining pheromone analog and only a small amount of the hydroxylactone. Exposure of this analog to water resulted in only 15% recovery after 20 hr, whereas the pheromone itself was stable under these conditions.

The Kovàts indices for the pheromone, Ia (2287), the trifluoroacetoxy analog, Ic (2071), and the heptadecafluoro analog, Id (1824), show a marked increase in volatility with increased fluorine substitution. The increase in volatility may contribute to the biological activity by enhancing aerial concentration. Nevertheless the activity of Id is an extremely revealing discovery in terms of pheromone receptor theory (Prestwich et al., 1990).

The synthesis strategy described has provided multigram quantities of

material for field use and revealed a surprising retention of biological activity with the introduction of seventeen fluorine atoms into the alkyl side chain.

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NEOTROPICAL ANT GARDENS
I. Chemical Constituents

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Abstract—In ant gardens of lowland Amazonia, parabiotic ant species *Camponotus femoratus* and *Crematogaster* cf. *limata parabiotica* cultivate a taxonomically diverse group of epiphytic plants, whose establishment is restricted to arboreal carton ant nests. Epiphyte seeds are collected by workers of *Ca. femoratus*, the larger of the two ants, and stored unharmed in brood chambers where they subsequently germinate. Although seeds of some ant-garden epiphytes bear nutritional rewards, previous studies have shown that these rewards are not sufficient to explain the pattern of ant attraction to seeds. Five aromatic compounds occur frequently in and on the seeds of most ant-garden epiphytes and may be chemical cues by which ants recognize propagules of their symbiotic plants. The most widely distributed of these is methyl 6-methylsalicylate [6-MMS] **1**, previously reported as a major mandibular gland product in related *Camponotus* species and present in trace quantities in *Ca. femoratus* males. (−)-Citronellol **6** (previously unreported in *Camponotus*) was the principal volatile constituent in extracts of male heads, and (−)-mellein **7** was present in small quantities. Discovery of 6-MMS inside the mandibular glands of male *Ca. femoratus* (and its presence in analogous glands of related ants) offers preliminary support for Ule's (1906) hypothesis that seeds attract ants by mimicking ant brood. In addition, the likely fungistatic activity of seed compounds suggests that they could retard microbial pathogens of ants and plants in the organic detritus of nest gardens. While the presence of identical seed compounds in so many unrelated plant lineages might represent a remarkable case of convergent evolution, other interpretations are possible.

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Key Words—Ant, ant garden, Hymenoptera, Formicidae, *Camponotus*, chemical mimicry, citronellol, convergent evolution, epiphyte, mellein, methyl 6-methylsalicylate, Perú, seed dispersal, symbiosis, tropical forest.

INTRODUCTION

In forests of lowland Amazonia, several ant species cultivate groups of unrelated epiphytic plants in arboreal carton nests. These "ant gardens" were discovered and named by Ule (1901) early in this century, and their descriptive natural history is now comparatively well known (Ule, 1905, 1906; Wheeler, 1921, 1942; Prance, 1973; Kleinfeldt, 1978, 1986; Madison, 1979; Davidson, 1988). Ants collect the seeds of symbiotic epiphytes and place these propagules inside the brood chambers of their nests. Workers also protect the plants from herbivore attack and "fertilize" their gardens by incorporating vertebrate feces into nest carton (Davidson and Epstein, 1989). Among specialized ant–plant associations, ant gardens are unique for their high diversity of taxonomically unrelated plants involved in the symbiosis. Our study is the first attempt to investigate a possible chemical basis for ant attraction to seeds of the many epiphyte species.

We studied ant-garden epiphytes from the Cocha Cashu Biological Station in the Manu National Park of Madre de Dios, Peru (11°52″S, 71°22″W, elevation $\simeq$400 m). In this seasonally inundated tropical moist forest, the alternation of wet and dry seasons drives the plant phenological cycles (Terborgh, 1983). Fruits of ant-garden epiphytes are located easily during annual fruiting peaks at the onset of the wet season in October. The following description of ant-garden natural history at Cocha Cashu is based on Davidson (1988).

Epiphytes restricted to ant gardens at Cocha Cashu include 10 species from seven plant families (Table 1). Interspecific differences in the abundance and distribution of plants in different gardens are correlated with differences in light regimes and successional stages of ant-garden development. However, the various ant-garden epiphytes occur in all possible combinations on individual gardens.

More than 90% of the ant-occupied gardens at Cocha Cashu contain the ant species *Camponotus femoratus* Fab. (Formicinae) and *Crematogaster* cf. *limata parabiotica* (Forel) (Myrmicinae). These ants cooperate in nest building and inhabit spatially segregated chambers of the same nests. Their "parabiotic" association could be mutualistic or parasitic, depending on the net outcome of the direct and indirect interactions through populations of jointly used plant and homopteran resources. Sweet exudates of phloem-feeding homoptera are the principle resources of these ants. Their Tupi name ("taracua," or "ants that feed on flowers") recognizes their tending of homoptera mainly on the pedicels of flowers and fruits, where phloem nutrients are especially concentrated.

TABLE 1. PERCENTAGES OF FIVE PREVALENT AROMATICS[a]

Seed sample	Compound 1	2	3	4	5
Anthurium gracile (Araceae) (Cocha Cashu 1984)	7.0	11.6			4.0
Anthurium ernestii (Araceae) (Cocha Cashu 1984)	2.0	28.6			
Anthurium ernestii (Araceae) (Cocha Cashu 1986)			T[b]		
Philodendron megalophyllum (Araceae) (Cocha Cashu 1984)	T	14.7			
Neoreglia sp. (Bromeliaceae) (Cocha Cashu 1984)	13.6		1.2	10.4	10.3
Streptocalyx longifolius (Bromeliaceae) (Cocha Cashu 1984)	23.6	T[c]			T[c]
Streptocalyx longifolius (Bromeliaceae) (Greenhouse 1986)	77.1	T[c]	T[c]	T	7.2
Epiphyllum phyllanthus (Cactaceae) (Cocha Cashu 1984)	19.3	8.8		4.0	
Epiphyllum phyllanthus (Cactaceae) (Greenhouse 1986)	7.3			6.8	
Codonanthe uleana (Gesneriaceae) (Cocha Cashu 1986)	8.2		T[c]		
Ficus paraensis (Moraceae) (Cocha Cashu 1984)		10.3			
Peperomia macrostachya (Piperaceae) (Cocha Cashu 1984)	25.8	4.8	10.6		T[c]
Peperomia macrostachya (Piperaceae) (Cocha Cashu 1985)	4.4		1.0		T[c]
Markei ulei (Solanaceae) (Cocha Cashu 1984)	14.0	11.1		T[c]	
Markei ulei (Solanaceae) (Greenhouse 1986)	74.0		T[c]	4.1	T[c]

[a] Values are the percentage of the component in the sample determined by GLC, assuming equal detector response for all components.
[b] T = trace; defined here as less than 1%.
[c] Determined by coinjection only.

Both ant species are polygynous (have multiple queens), and colonies fission as they grow, generally moving to track areas of relatively high resource concentration. Aggregations of ant gardens formed by fissioning occupy 16–39% of the Cocha Cashu forests, depending on habitat type. Epiphytes are propagated in the new gardens by incorporating seeds into ant carton early in nest construction, but seeds are added to the gardens continuously throughout

their lifetimes. The larger species, *Ca. femoratus*, is responsible for collecting most of the thousands of seeds that accumulate in the galleries of even small nests. Smaller workers of *Cr.* cf. *limata parabiotica* can only move epiphyte seeds over distances of a few centimeters.

The strongly correlated occurrences of epiphyte and ant populations suggest that the groups are mutualistic at the population level and that they may even be obligate mutualists. Nevertheless, there is currently no direct evidence that either ants or plants have evolved special adaptations to their associations with one another. Four observations from field studies suggested that specialized chemical cues may attract ants to epiphyte seeds (Davidson, 1988): (1) Seeds of several different ant-garden epiphytes smell strongly of volatile, vanillinlike compounds. (2) Unspecialized congeners of ant-garden ants are repelled by contact with the seeds of ant-garden epiphytes. This is true even for seeds with obvious nutritional appendages (arils or elaiosomes). (3) In cafeteria trials, where seeds of several ant-garden plants were offered simultaneously to *Ca. femoratus*, workers expressed a significant preference ranking for seeds (*Codonanthe uleana* > *Anthurium ernestii* > *Anthurium gracile* > *Markei ulei* > *Streptocalyx longifolius* > *Philodendron megalopyllum* > *Ficus paraensis* > *Peperomia macrostachya*). This preference order corresponds poorly to rankings of either the nutritional rewards on individual seeds or of the expected resource gains from established plants. (4) Seeds of one ant-garden epiphyte were discriminated successfully from those of a congeneric plant species that does not live in ant gardens, even after nutritional fruit pulp was removed by transit through the digestive tracts of frugivorous bats.

Collectively, this evidence suggests that seed recognition cues may be based on volatile chemical signals, rather than nutritional rewards. Here, we describe our chemical analyses of both epiphyte seed compounds and exocrine products of *Ca. femoratus*, the principal seed disperser. Field assays of ant behavior in relation to seed compounds will be presented in a second report.

METHODS AND MATERIALS

Extractive Methods

Native Epiphyte Seeds and Fruits. Seed and fruit specimens were collected during September through November of 1984, 1985, and 1986 from ant gardens located near the Cocha Cashu Biological Station in the Manu National Park of Madre de Dios, Peru. The collected plant materials were transferred immediately to glass receptacles containing 70% ethanol–water (to inhibit biological degradation) for transport to Salt Lake City. Extracts of seeds and fruits were prepared in a similar manner for all epiphyte species. The details of a typical example are presented here.

Anthurium gracile seeds were collected during September through November 1984 at Cocha Cashu and stored in 70% ethanol until analyzed. The ethanol solution (6 ml) was decanted from the seeds, the seeds were extracted with 3 × 10 ml of pentane, and the combined extracts were set aside. Pentane (10 ml) was added to the seeds, and the mixture was allowed to stand at room temperature for 24 hr. The solvent was decanted and combined with the previous extracts. The cycle of extraction was repeated twice. Seeds were ground in a blender, and the extraction cycle with pentane was repeated five and seven days later. The combined pentane extracts were concentrated in vacuo at 0°C and nonequilibrium distilled at a maximum temperature of 125°C (0.20 torr) to give a colorless oil which was used for GLC-MS analysis and GLC coinjection studies.

In some experiments the supernatant (70% ethanol solution) and seeds, ground or unground, were extracted using continuous extraction, or in the case of solids, a Soxhlet with pentane as solvent, and analyzed separately (Tables 1–3) using the same work-up as above.

Greenhouse Epiphyte Seeds and Fruits. Additional seed and fruit samples were obtained from epiphytes grown from seed in the University of Utah tropical greenhouse. These samples were studied in a manner similar to those collected at Cocha Cashu, except that fresh material was never in contact with ethanol (Tables 1 and 2). Furthermore, a series of extractions was undertaken

TABLE 2. SAMPLES CONTAINING MONOTERPENES[a]

Seed sample	α-Pinene	β-Pinene	Limonene	*trans*-Pinocarveol
Anthurium ernestii (Cocha Cashu 1984)			T[b,c]	
Anthurium ernestii (Cocha Cashu 1986)	T[c] (1.3)	T[c] (1.3)	2.5 (0.9)	
Neoreglia sp. (Cocha Cashu 1986)	(1.3)	(4.1)		
Epiphyllum phyllanthus (Utah greenhouse 1986)				2.5
Peperomia macrostachya (Cocha Cashu 1986)			5.4	
Peperomia macrostachya (Utah greenhouse 1986)			1.5[c]	

[a] Values are the percentage of the component in the sample determined by GLC, assuming equal detector response for all components. Values in parentheses are from supernatants used to store the seeds during shipment from Cocha Cashu.
[b] T = trace; defined in this case as less than 1%.
[c] Determined by coinjection only.

TABLE 3. SAMPLES CONTAINING AROMATICS[a]

Seed sample	ME	EE	UA	PE	2H5MB	3H2MB
Ficus paraensis (Cocha Cashu 1984)				1.2	1.2	7.0
Peperomia macrostachya (Cocha Cashu 1984)	1.6		22.4			
Peperomia macrostachya (Cocha Cashu 1985)	1.5	1.4	T[b]			
Peperomia macrostachya (Cocha Cashu 1986)	10.7		2.7			

[a]Values are the percentage of the component in the sample determined GLC, assuming equal detector response for all components. Values in parentheses are from supernatants used to store the seeds during shipment from Cocha Cashu. Abbreviations are as follows: ME = 2-hydroxybenzoic acid, methyl ester, EE = 2-hydroxybenzoic acid, ethyl ester, UA = unknown aromatic with *m/z* of 152, PE = 1-(2-hydroxy-4-methylphenyl)ethanone, 2H5MB = 2-hydroxy-5-methoxybenzaldehyde, 3H2MB = 3-hydroxy-2-methoxybenzaldehyde.
[b]T = trace; defined in this case as less than 1%.

on these materials to assess the pattern of occurrence of seed components within the different plant tissues. Fruit pulp and juice extracts were analyzed separately for seeds of *S. longifolius*, *E. phyllanthus*, and *M. ulei*. To look for the presence of seed compounds in the coats of *S. longifolius* seeds, the seeds were washed repeatedly with pentane, then ground, and continuously extracted with pentane (Table 4). Finally, whole seeds of *M. ulei* were continuously extracted with pentane for four days in an effort to dissolve any seed compounds localized in the seed coat. The seeds were then ground, reextracted, and the extracts analyzed to explore the possibility of seed components being housed in the inner seed.

Male Camponotus femoratus *Ants.* A common component of seed and fruit extracts (**1**) has been reported previously in male *Camponotus* ants (Brand et al., 1973a,b; Payne et al., 1975; Blum et al., 1987). We analyzed *Camponotus femoratus* males to determine if **1** or other seed and fruit components occur in this species. Furthermore, to establish the sites of production of ant compounds, we analyzed heads separately from the combined thorax and gaster.

A sample of 350 male *Camponotus femoratus* ant heads collected during March 1987 at Reserva Tambopata (near Cocha Cashu) was stored in CH_2Cl_2 (10 ml). The CH_2Cl_2 phase was removed, passed through a micropore filter, concentrated in vacuo at 0°C to 1 ml, and the resulting oil was subjected to GLC-MS analysis. A portion of the CH_2Cl_2 extract was set aside (0.05 ml), and the remainder (0.95 ml) was placed on an 8 × 20-cm preparative TLC plate and eluted with 35% (v/v) EtOAc–Hex. A non-UV-active compound and

TABLE 4. MISCELLANEOUS SAMPLES CONTAINING THE FIVE PREVALENT AROMATICS[a]

Sample	Compound 1	2	3	4	5
Streptocalyx longifolius untreated seeds (Utah greenhouse 1986)	71.7	T[b,c]	T[c]	T	7.2
Streptocalyx longifolius pentane washed seeds (Utah greenhouse 1986)	44.4			1.9	40.1
Streptocalyx longifolius fruit juice and pulp (Utah greenhouse 1986)	57.5	1.7	T	26.2	6.8
Epiphyllum phyllanthus untreated seeds (Utah greenhouse 1986)	7.3			6.8	
Epiphyllum phyllanthus fruit juice and pulp (Utah greenhouse 1986)	T			18.0	
Markei ulei whole untreated seeds (Utah greenhouse 1986)	65.8		T	T	16.0
Markei ulei ground preextracted seeds (Utah greenhouse 1986)	T[c]		T[c]	T[c]	T[c]
Markei ulei fruit juice and pulp (Utah greenhouse 1986)	4.8		T[c]	3.3	23.2

[a] Values are the percentage of the component in the sample determined by GLC, assuming equal detector response for all components.
[b] T = trace; defined in this case as less than 1%.
[c] Determined by coinjection only.

a UV-active compound were removed from the plate using ether, the ethereal extracts concentrated in vacuo, and the compounds analyzed by [^{1}H]NMR, CD, and ORD.

The 350 heads were ground to a fine powder at −196°C, then continuously extracted with pentane (125 ml) for five days. The extract was concentrated in vacuo at 0°C, passed through a micropore filter, and the resulting oil was subjected to GLC-MS analysis.

A sample of 100 gasters and thoraxes from the males whose heads were extracted above was stored in CH_2Cl_2 (2 ml). The CH_2Cl_2 phase was separated, passed through a micropore filter, concentrated in vacuo to a volume of 0.1 ml, and subjected to GLC analysis.

Coinjection Studies. An examination of the preliminary GLC-MS data from the plant extracts suggested the presence of a number of aromatic and terpene compounds. A standard consisting of 37 compounds (Table 5) was prepared, and suitable conditions were developed for capillary GLC analysis of both plant and ant extracts. Because clear distinctions could not be made by MS between isomers of some components, all reasonable isomers were included.

TABLE 5. STANDARD COMPOUNDS USED FOR COINJECTION STUDIES[a]

Monoterpenes
- α-Pinene
- β-Pinene
- Limonene
- Linalool
- Citronellal
- Citronellol
- Citral A and B
- *trans*-Pinocarveol (**9**)

Benzenemethanols
- 2-Hydroxy-3-methoxybenzenemethanol (**10**)

Benzaldehydes
- 2-Hydroxy-3-methoxybenzaldehyde
- 2-Hydroxy-4-methoxybenzaldehyde (**11**)
- 2-Hydroxy-5-methoxybenzaldehyde (**12**)
- 2-Hydroxy-6-methoxybenzaldehyde (**16**)
- 3-Hydroxy-2-methoxybenzaldehyde (**19**)
- 3-Hydroxy-4-methoxybenzaldehyde
- 3-Hydroxy-5-methoxybenzaldehyde (**23**)
- 4-Hydroxy-2-methoxybenzaldehyde (**26**)
- 4-Hydroxy-3-methoxybenzaldehyde
- 5-Hydroxy-2-methoxybenzaldehyde (**27**)

Benzoic acid esters
- 2-Aminobenzoic acid, methyl ester
- 2-Hydroxybenzoic acid, methyl ester
- 2-Hydroxybenzoic acid, ethyl ester (**28**)
- 2-Hydroxy-3-methylbenzoic acid, methyl ester (**29**)
- 2-Hydroxy-4-methylbenzoic acid, methyl ester (**30**)
- 2-Hydroxy-5-methylbenzoic acid, methyl ester (**31**)
- 2-Hydroxy-6-methylbenzoic acid, methyl ester (**1**) (6-MMS)

Phenylethanones
- 1-(2-Hydroxy-3-methylphenyl)ethanone (**32**)
- 1-(2-Hydroxy-4-methylphenyl)ethanone (**33**)
- 1-(2-Hydroxy-5-methylphenyl)ethanone (**34**)
- 1-(2-Hydroxy-6-methylphenyl)ethanone (**4**)
- 1-(2-Acetyloxy-6-methylphenyl)ethanone (**35**)
- 1-(2,4-Dihydroxyphenyl)ethanone
- 1-(2,5-Dihydroxyphenyl)ethanone
- 1-(2,6-Dihydroxyphenyl)ethanone

Phenols
- Eugenol
- Isoeugenol

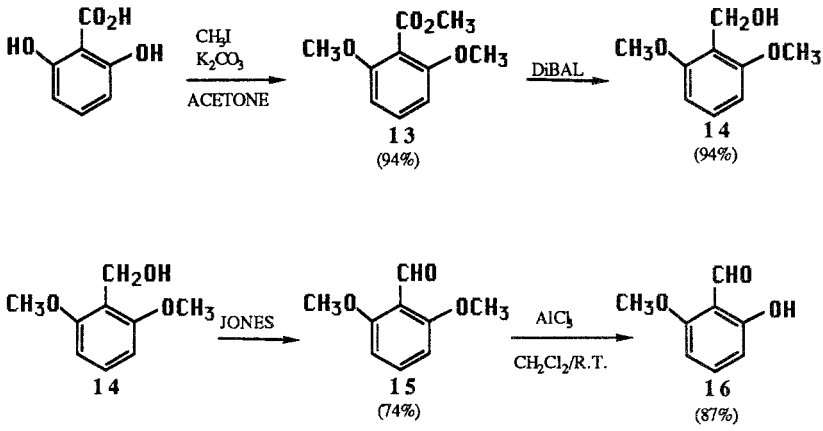
[a] Compounds with numbers assigned were prepared as indicated in Methods and Materials. All other compounds were obtained from commercial suppliers.

Instrumentation and Materials. IR spectra were obtained with a Perkin-Elmer 298, Perkin-Elmer 1500, or Bio-Rad (Digilab) FTS-40 spectrophotometer with absorptions reported in wavenumbers (cm^{-1}) and relative intensities reported as strong (s) (0–33% transmittance), medium (m) (34–66% transmittance), or weak (w) (67–100% transmittance). [^{1}H]NMR were recorded on a Varian EM-390 or XL-300 spectrometer with chemical shifts given in parts per million relative to tetramethylsilane (δ 0). Mass spectrometric (MS) determinations were carried out on a VG Analytical 7070E double-focusing mass spectrometer at 70 eV unless otherwise noted and are reported as *m/z* (relative intensity). A Hewlett-Packard model HP5840A gas chromatograph with a 30-m × 0.32-mm-id fused silica DB-5 (1-μm-thick coating) capillary column with a 20:1 split ratio was interfaced to the mass spectrometer for obtaining GLC-MS spectra. An 80°C to 250°C at 5°C/min temperature program, with a 20-min final temperature hold time, was utilized during GLC-MS runs. A Varian series 3400 gas chromatograph with a flame-ionization detector and Varian Vista series 402 data station were employed for capillary GLC analyses and coinjection studies using an identical capillary column and temperature program as for

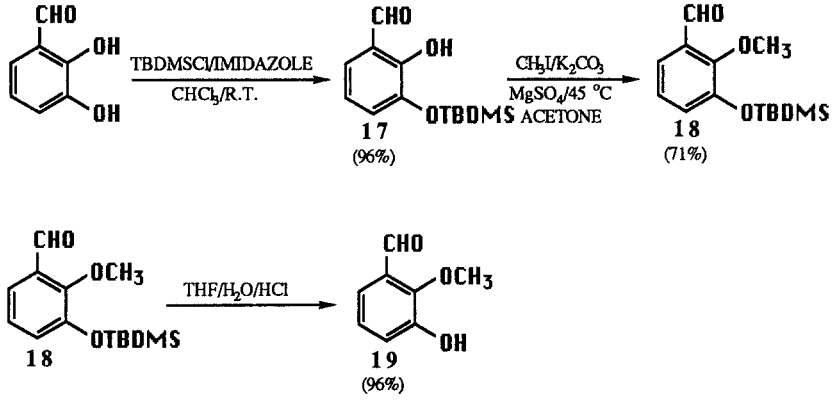
GLC-MS analyses. Circular dichroism (CD) and optical rotary dispersion (ORD) spectra were recorded on a Jasco model J-20C spectrophotometer using a 0.1-cm microcell. UV spectra were recorded on a Perkin-Elmer 552A spectrophotometer using a 1.0-cm cell. Melting points were obtained on a Mel Temp melting point apparatus and are uncorrected. Preparative thin-layer chromatography (preparative TLC) was performed on precoated sheets (0.5 mm thick) of silica gel Si500F (Baker) with UV detection. Thin-layer chromatography analyses were performed on precoated sheets (0.2 mm thick) of silica gel 60F (E. Merck) with detection by UV, phosphomolybdic acid, or 2,4-dinitrophenylhydrazine reagent. Flash chromatography was performed on 230–400 mesh ASTM silica gel 60 (EM reagents). Medium pressure liquid chromatography (MPLC) was performed on 230–400 mesh ASTM silica gel 60 (EM reagents) using a Milton Ray mini-pump and Altex columns. Ethyl acetate (EtOAc) and hexane (Hex) were distilled prior to use and spectral grade solvents were used for spectoscopic measurements. Tetrahydrofuran (THF), ether, and toluene were freshly distilled from potassium–benzophenone ketal. Dichloromethane (CH_2Cl_2) was freshly distilled from calcium hydride. Methanol (MeOH) was distilled from magnesium and stored over 4 Å molecular sieves. All reactions were run under nitrogen or argon atmospheres unless otherwise noted.

Synthesis of Coinjection Standards

The syntheses of the standards were accomplished readily via one-step reactions from commercially available starting materials, with the exception of compounds **16, 19, 23,** and **26,** whose syntheses are outlined in Schemes 1–4. Compounds **1** and **4** were synthesized from **8.** Figure 1 shows the structures of compounds **1–7.**

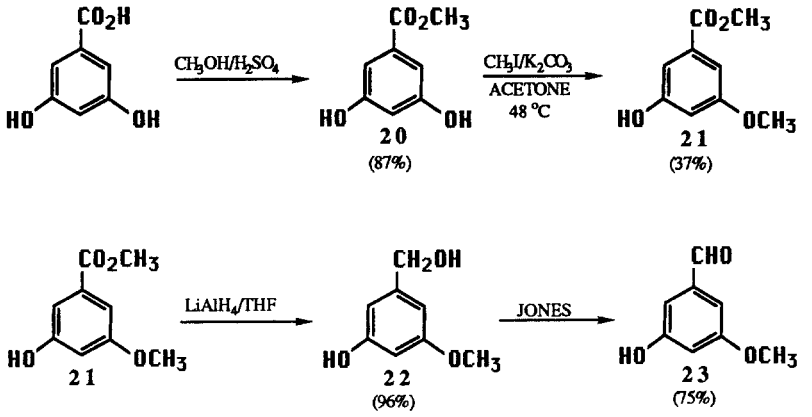


SCHEME 1. Synthesis of compound **16.**

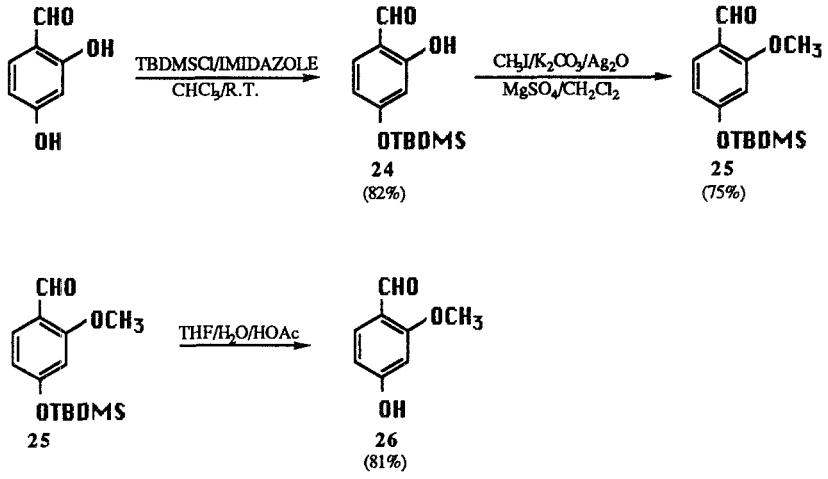


SCHEME 2. Synthesis of compound **19.**

2-Hydroxy-6-methylbenzoic Acid, Methyl Ester ***(1).*** To a stirred solution of 2.0 g (13.1 mmol) of **8** in 20 ml of MeOH was slowly added 3.0 ml of conc. H_2SO_4. The reaction mixture was heated to reflux and stirred 18 hr. After adding water (20 ml) and cooling to room temperature, the reaction mixture was extracted with ether (3 × 25 ml). The combined extracts were washed with sat. aq. $NaHCO_3$ (2 × 10 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by flash chromatography on a 2.5 × 10-cm column using 10% (v/v) EtOAc–Hex gave 2.0 g (92%) of **1** as transparent, rhombic crystals: mp 30.5–31.5°C; lit 30.5–31.0°C (Eliel et al., 1953); R_f 0.60 (35% (v/v) EtOAc–Hex); IR (melt) 3500–2700 m, 1650 s, 800 s, 745 s, 695 s; [^{1}H]NMR ($CDCl_3$) 2.49 (s, 3H), 3.90 (s, 3H), 6.61 (d, J = 8 Hz, 1H), 6.73 (d, J = 8 Hz, 1H), 7.17



SCHEME 3. Synthesis of compound **23.**



SCHEME 4. Synthesis of compound **26.**

(dd, J = 8 Hz, 1H), 11.23 (s, 1H); MS, 166 (35), 135 (26), 134 (100), 106 (31), 105 (17), 78 (21), 77 (18), 51 (13).

1-(2-Hydroxy-6-methylphenyl)ethanone ***(4).*** To a stirred solution of 2.00 g (13.1 mmol) of **8** in 100 ml of ether at 0°C was added 30.0 ml (45.0 mmol) of 2 M MeLi (in ether) over 15 min. The reaction mixture was warmed to room temperature, stirred for 24 hr, added to a stirred solution of 200 g of crushed ice in 100 ml of 10% HCl solution in 2 to 3-ml portions, and extracted with ether (3 × 50 ml). The combined organic extracts were extracted with sat. aq. $NaHCO_3$ (3 × 25 ml) to recover starting material. After drying the combined

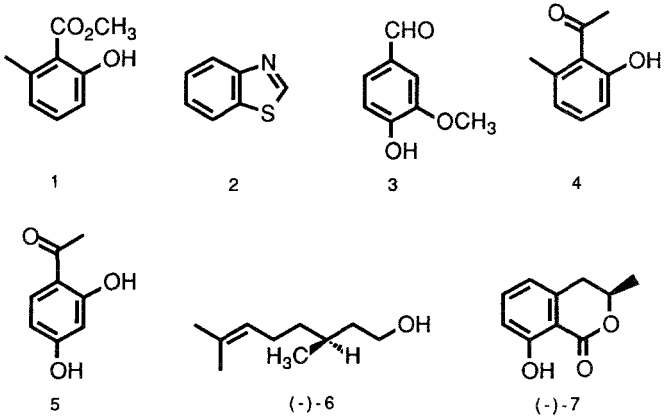


FIG. 1. Structures of compounds **1–7.**

organic extracts over $MgSO_4$, concentrating in vacuo, and purifying by flash chromatography on a 1 × 20-cm column using 35% (v/v) EtOAc–Hex, 1.28 g (65%) of **4** was obtained as a light yellow oil: Lit reported as an oil (Cremer and Tarbell, 1961; Jones et al., 1981); R_f 0.50 [35% (v/v) EtOAc–Hex]; IR (neat) 3650–2300 s, 1675 s, 780 s, 730 s; [^{1}H]NMR ($CDCl_3$) 2.54 (s, 3H), 2.62 (s, 3H), 6.69 (dd, J = 8.5 Hz, 2H), 7.18 (t, J = 8.5 Hz, 1H), 12.17 (s, 1H); MS 150 (92), 136 (100), 107 (11), 79 (16), 78 (13), 77 (24), 51 (16), 43 (42). Acidification of the bicarbonate extracts and recrystallization from boiling water gave 0.65 g of the starting acid **8.** Overall yield of **4** based on recovered **8** was 96%.

2-Hydroxy-6-methylbenzoic Acid ***(8).*** An open air solution of 5.0 g (6.62 mmol) of 2-amino-6-methylbenzoic acid (Aldrich) and 12.5 g of conc. H_2SO_4 in 50 ml of water was stirred until the amino acid had dissolved. The reaction mixture was cooled to 0°C and a solution of 3.0 g (8.70 mmol) of $NaNO_2$ in 50 ml of water added dropwise. After stirring 1 hr at 0°C, the diazonium salt was decomposed by slowly adding the reaction mixture to a boiling solution of 20 ml of conc. H_2SO_4 in 200 ml of water at a rate that prevented excessive foaming or cessation of boiling. The solution was cooled to room temperature, then to 0°C before suction filtration. Recrystallization from boiling water (350 ml) with decolorizing carbon gave 2.7 g (54%) of **8** as long, off-white needles: mp 173–174°C; lit 168°C (Asahina and Kondo, 1922), 170–171°C (Eliel et al., 1953); R_f 0.15 [35% (v/v) EtOAc–Hex]; IR (mineral oil) 3500–2200 s, 1690 s, 800 s; [^{1}H]NMR ($CDCl_3$–acetone-d_6) 2.56 (s, 3H), 6.69 (d, J = 8.5 Hz, 1H), 6.72 (d, J = 8.5 Hz, 1H), 7.20 (dd, J = 8.5 Hz, 1H), 9.40 (br s, 2H); MS 152 (33), 135 (12), 134 (100), 106 (45), 105 (36), 78 (47), 77 (24), 51 (25).

*(+)-*trans*-Pinocarveol* ***(9).*** Using the procedure of Hartshorn and Wallis (1964), 10.22 g (11.9 ml, 75 mmol) of (1*S*)-(−)-β-pinene ($[\alpha]_D^{20}$ − 21 [neat] (Aldrich) was transformed into 2.13 g (37% of the theoretical, based on the limiting reagent) of (+)-9. The desired product was obtained as a clear oil with the following properties: $[\alpha]_D^{20}$ + 56.87 (c 21.0, $CHCl_3$); lit $[\alpha]_D^{20}$ − 73.7 (c 5, ethanol) (Schmidt, 1944); R_f 0.36 [20% (v/v) EtOAc–Hex]; IR (neat) 3600–3100 s, 3070 m, 1645 m, 1020 s, 998 s, 890 s; [^{1}H]-NMR ($CDCl_3$) 0.64 (s, 3H), 1.27 (s, 3H), 1.62–2.68 (m, 7H), 4.44 (d, J = 8 Hz, 1H), 4.85 (s, 1H), 5.04 (s, 1H); MS 152 (1), 134 (44), 109 (32), 92 (100), 91 (59), 83 (81), 81 (34), 70 (90), 69 (48), 55 (76), 41 (79).

2-Hydroxy-3-methoxybenzenemethanol ***(10).*** To a stirred solution of 1.5 g (39.4 mmol) of $LiAlH_4$ in 50 ml of ether at 0°C was slowly added a solution of 5 g (32.9 mmol) of 2-hydroxy-3-methoxybenzaldehyde (Aldrich) in 30 ml of ether. The reaction mixture was warmed to room temperature and stirred 2 hr. After cooling to 0°C, the reaction mixture was quenched with 3 N H_2SO_4 and extracted with ether (2 × 25 ml). The combined ethereal extracts were

dried over $MgSO_4$ and concentrated in vacuo. Purification by flash chromatography on a 3 × 20-cm column using 35% (v/v) EtOAc–Hex gave 3.3 g (65%) of **10** as a white solid: mp 60–61°C; lit mp 61–62°C (Eliel, 1951); R_f 0.23 [35% (v/v) EtOAc–Hex]; IR (mineral oil) 3550–2750 s, 1075 s, 1020 s, 770 s, 730 s; [^{1}H]NMR ($CDCl_3$–acetone-d_6) 3.82 (s, 3H), 4.00 (br s, 1H), 4.69 (s, 2H), 6.78–6.94 (m, 3H), 7.27 (br s, 1H); MS 154 (46), 136 (100), 135 (50), 107 (48), 106 (43), 105 (20), 93 (26), 78 (22), 65 (67), 39 (30).

2-Hydroxy-4-methoxybenzaldehyde ***(11).*** Using the procedure of Mali et al. (1982), 2.00 g (12.0 mmol) of 2,4-dimethoxybenzaldehyde (Aldrich) was selectively demethylated to give 1.75 g (96%) of **11** as an off-white solid: mp 40–41°C; lit 40°C (Mali et al., 1982); R_f 0.30 [35% (v/v) EtOAc–Hex]; IR (thin film) 3600–2800 s, 1631 s; [^{1}H]NMR ($CDCl_3$) 3.83 (s, 3H), 6.35–6.68 (m, 2H), 7.39 (d, J = 9 Hz, 1H), 9.78 (s, 1H), 11.50 (s, 1H); MS 152 (64), 151 (100), 108 (10), 95 (14), 63 (11), 53 (12).

2-Hydroxy-5-methoxybenzaldehyde ***(12).*** Using the same procedure as noted for the preparation of **11,** 2.00 g (12.0 mmol) of 2,5-dimethoxybenzaldehyde (Aldrich) was selectively demethylated to give 0.74 g (40%) of **12** as a light yellow oil: lit bp 247–248°C (Tiemann and Müller, 1881); R_f 0.32 [35% (v/v) EtOAc–Hex]; IR (neat) 3600–2900 m, 3010 m, 2740 m, 2640 m, 1710 s; [^{1}H]NMR ($CDCl_3$) 3.78 (s, 3H), 6.78–7.20 (m, 3H), 9.78 (s, 1H), 10.69 (s, 1H); MS 152 (100), 137 (77), 109 (25), 81 (26), 53 (37).

2,6-Dimethoxybenzoic Acid, Methyl Ester ***(13).*** To a stirred solution of 10.0 g (64.9 mmol) of 2,6-dihydroxybenzoic acid (Aldrich) and 54.0 g (390 mmol) of anhyd. K_2CO_3 in 100 ml of acetone was added 46.1 g (20.2 ml, 325 mmol) of CH_3I. The reaction mixture was heated to a gentle reflux and stirred 36 hr. The reaction mixture was concentrated in vacuo, diluted with water (150 ml), and extracted with ether (3 × 75 ml). The combined extracts were washed with sat. aq. $NaHCO_3$ (50 ml), dried over $MgSO_4$, and concentrated in vacuo to give 12.0 g (94%) of **13** as white needles: mp 86–87°C; lit 88°C (Clewer et al., 1915); R_f 0.35 [35% (v/v) EtOAc–Hex]; IR (thin film) 1730 s; [^{1}H]NMR ($CDCl_3$) 3.85 (s, 6H), 3.94 (s, 3H), 6.60 (d, J = 12.5 Hz, 1H), 6.70 (s, 1H), 7.37 (dd, J = 12.5 Hz, 1H); MS 196 (38), 165 (100), 150 (8), 107 (7), 166 (5), 164 (4), 136 (3), 135 (4), 122 (3).

2,6-Dimethoxybenzenemethanol ***(14).*** To a stirred solution of 10.6 g (54.0 mmol of **13** in 100 ml of CH_2Cl_2 at −78°C was added 45.0 ml (67.5 mmol) of 1.5 M DiBAL (in toluene) over 0.5 hr. The reaction mixture was stirred 16 hr at −78°C before slowly adding 20 ml of MeOH. The reaction mixture was warmed to room temperature and sat. aq. Rochelle salt solution (150 ml) added. After stirring overnight, the reaction mixture was extracted with CH_2Cl_2 (2 × 50 ml). The combined extracts were washed with water (50 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by flash chromatography on a 2.5 × 10-cm column using 35% (v/v) EtOAc–Hex gave 8.5 g (94%) of **14** as

a white solid: mp 53–54°C; lit 54–56°C (Bowie et al., 1967); R_f 0.27 [35% (v/v) EtOAc–Hex]; IR (melt) 3550–3100 s, 995 s, 765 s; [^{1}H]NMR ($CDCl_3$) 2.48 (t, J = 6 Hz, 1H), 3.80 (s, 6H), 4.77 (d, J = 6 Hz, 2H), 6.52 (d, J = 8 Hz, 2H), 7.13 (t, J = 8 Hz, 1H); MS 168 (100), 167 (19), 151 (14), 137 (15), 136 (52), 135 (18), 107 (18), 91 (17), 77 (15).

2,6-Dimethoxybenzaldehyde ***(15).*** To a stirred open air solution of 8.5 g (50.5 mmol) of **14** in 50 ml of acetone at 0°C was slowly added Jones reagent until the color persisted. The reaction mixture was stirred for 5 min, diluted with water (200 ml), and extracted with ether (3 × 50 ml). The combined extracts were washed with sat. aq. $NaHCO_3$ (2 × 50 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by flash chromatography on a 2.5 × 10-cm column using 35% (v/v) EtOAc–Hex, followed by recrystallization from EtOAc–Hex gave 6.0 g (71%) of **15** as off-white platelets: mp 97–98°C; lit 96°C (Martens and Praefuke, 1974), 97–98°C (Mauthner, 1919); R_f 0.20 [35% (v/v) EtOAc–Hex]; IR (thin film) 1680 s; [^{1}H]NMR ($CDCl_3$) 3.84 (s, 6H), 6.52 (d, J = 8 Hz, 2H), 7.39 (t, J = 8 Hz, 1H), 10.47 (s, 1H); MS 166 (100), 165 (24), 150 (11), 149 (15), 148 (11), 107 (9), 90 (5), 77 (7), 76 (20).

2-Hydroxy-6-methoxybenzaldehyde ***(16).*** Using the same procedure as noted for the preparation of **11,** 2.00 g (12.0 mmol) of **15** was selectively demethylated to give 1.60 g (87%) of **16** as light yellow needles: mp 74–75°C; lit 74–75°C (Schamschurin, 1944); R_f 0.41 [35% (v/v) EtOAc–Hex]; IR (thin film) 3500–2700 m, 1650 s, 1621 s, 761 s, 706 s; [^{1}H]NMR ($CDCl_3$) 3.84 (s, 3H), 6.32 (d, J = 8 Hz, 1H), 6.49 (d, J = 8 Hz, 1H), 7.37 (t, J = 8 Hz, 1H), 10.28 (s, 1H), 11.94 (s, 1H); MS 152 (100), 151 (44), 134 (58), 106 (37), 92 (48), 78 (35), 65 (25), 53 (27), 39 (27).

3-[(tert-Butyldimethylsily)oxy]-2-hydroxybenzaldehyde ***(17).*** To a stirred solution of 8.2 g (54.3 mmol) of *tert*-butylchlorodimethylsilane in 100 ml of $CHCl_3$ was added 3.7 g (54.3 mmol) of imidazole. After 5 min, 5.0 g (36.2 mmol) of 2,3-dihydroxybenzaldhyde (Aldrich) was added and the reaction mixture stirred at room temperature for 8 hr. The reaction mixture was washed with water (2 × 25 ml) and brine (20 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by MPLC on a 5 × 50-cm column using a gradient of 10% to 20% (v/v) EtOAc–Hex gave 8.8 g (96%) of **17** as a yellow oil: R_f 0.64 [35% (v/v) EtOAc–Hex]; IR (neat) 3500–2400 m, 1652 s; [^{1}H]NMR ($CDCl_3$) 0.21 (s, 6H), 1.00 (s, 9H), 6.72–7.26 (m, 3H), 9.88 (s, 1H), 10.93 (s, 1H); MS [no M^+], 196 (8), 195 (100), 167 (58), 151 (2), 75 (3).

3-[(tert-Butyldimethylsilyl)oxy]-2-methoxybenzaldehyde ***(18).*** To a stirred solution of 2.0 g (7.9 mmol) of **17** and 2.7 g (19.8 mmol) of anhyd K_2CO_3 in 20 ml of acetone at 45°C was added 2.3 g (1.0 ml, 15.9 mmol) of CH_3I. After 2.5 hr, the reaction mixture was diluted with water (20 ml) and extracted with ether (3 × 20 ml). The combined extracts were washed with 10% NaOH (3 × 7 ml), sat. $NaHSO_3$ (10 ml), and brine (10 ml), dried over $MgSO_4$, and con-

centrated in vacuo. Purification by MPLC on a 2.5 × 100-cm column using 20% (v/v) EtOAc–Hex gave 1.5 g (71%) of **18** as a clear, near-colorless oil: R_f 0.25 [5% (v/v) EtOAc–Hex]; IR (neat) 1660 s, 745 s; [^{1}H]NMR ($CDCl_3$) 0.22 (s, 6H), 1.02 (s, 9H), 3.90 (s, 3H), 7.04 (d, J = 7 Hz, 1H), 7.08 (d, J = 3 Hz, 1H), 7.42 (dd, J = 3, 7 Hz, 1H), 10.38 (s, 1H); MS (CH_4 chemical ionization) 267 (99), 265 (11), 251 (8), 225 (6), 210 (12), 209 (100), 194 (29).

3-Hydroxy-2-methoxybenzaldehyde ***(19).*** A stirred solution containing 2.30 g (8.6 mmol) of **18,** 15 ml of THF, and 2.0 ml of conc. HCl was stirred at room temperature in the dark for 8 hr. The reaction mixture was diluted with water (15 ml) and extracted with ether (3 × 20 ml). The combined extracts were washed with water (10 ml), sat. aq. $NaHCO_3$ (2 × 10 ml) and brine (10 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by flash chromatography on a 5 × 20-cm column using 20% (v/v) EtOAc–Hex gave 1.26 g (96%) of **19** as a white solid: mp 113.5–114.0°C; lit 113–115°C (Funke and Gombert, 1959); R_f 0.38 [35% (v/v) EtOAc–Hex]; IR (thin film) 3500–2400 s, 1660 s; [^{1}H]NMR ($CDCl_3$–acetone-d_6) 3.96 (s, 3H), 6.87–7.37 (m, 3H), 8.14 (s, 1H), 10.32 (s, 1H); MS 152 (100), 137 (13), 134 (12), 109 (18), 106 (14), 92 (15), 81 (16), 53 (10).

3,5-Dihydroxybenzoic Acid, Methyl Ester ***(20).*** Using a procedure similar to the one used in preparing **1,** 20.0 g (130 mmol) of 3,5-dihydroxybenzoic acid (Aldrich) was methylated to give 19.0 g (87%) of **20** as an off-white solid: mp 167.0–167.5°C; lit 165°C (Birkinshaw and Bracken, 1942); R_f 0.15 [35% (v/v) EtOAc–Hex]; IR (thin film) 3500–3000 s, 1695 s; [^{1}H]NMR (acetone-d_6) 3.80 (s, 3H), 6.53 (t, J = 2 Hz, 1H), 6.95 (d, J = 2 Hz, 2H), 8.35 (br s, 2H): MS 168 (68), 138 (5), 137 (100), 110 (6), 109 (39), 81 (7), 69 (11), 53 (4), 51 (4).

3-Hydroxy-5-methoxybenzoic Acid, Methyl Ester ***(21).*** To a stirred solution of 5.0 g (29.7 mmol) of **20** and 10.3 g (74.4 mmol) of anhyd. K_2CO_3 in 50 ml of acetone at 48°C was added 8.4 g (3.7 ml, 59.5 mmol) of CH_3I. The reaction mixture was stirred at 48°C for 1 hr, diluted with water (100 ml), cooled to room temperature, and extracted with ether (3 × 50 ml). The combined extracts were washed with brine (25 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by MPLC on a 5 × 50-cm column using 20% (v/v) EtOAc–Hex gave 2.0 g (37%) of **21:** mp 94.5–95.0°C; lit 92.5–93.5°C (Danishefsky et al., 1978); R_f 0.30 [35% (v/v) EtOAc–Hex]; IR (thin film) 3600–3200 s, 1696 s; [^{1}H]NMR ($CDCl_3$) 3.75 (s, 3H), 3.85 (s, 3H), 6.72 (t, J = 2.5 Hz, 1H), 7.04–7.28 (m, 3H); MS 182 (93), 152 (6), 151 (100), 124 (12), 123 (40), 108 (10), 93 (6), 69 (12), 51 (5).

3-Hydroxy-5-methoxybenzenemethanol ***(22).*** To a stirred solution of 0.62 g (16.4 mmol of $LiAlH_4$ in 50 ml of THF at 0°C was slowly added 2.00 g (11.0 mmol) of **21** in small portions. The reaction mixture was warmed to room temperature, stirred for 12 hr, cooled to 0°C, and quenched with 10% HCl (10

ml). The reaction mixture was filtered through diatomaceous earth and diluted with brine (20 ml). The organic phase was separated, dried over $MgSO_4$, and concentrated in vacuo to give 1.63 g (96%) of **22** as a viscous light yellow oil: lit mp 85–86°C (Elix and Ferguson, 1978); R_f 0.18 [45% (v/v) EtOAc–Hex]; IR (neat) 3650–2500 s, 832 s.

3-Hydroxy-5-methoxybenzaldehyde ***(23).*** Using a procedure similar to the one used in preparing **16,** 1.63 g (10.6 mmol) of **22** was oxidized to give 1.20 g (75%) of **23** as an off-white solid: mp 128.5–129.0°C; lit 130–131°C (Mauthner, 1927; Spath and Kromp, 1941); R_f 0.37 [45% (v/v) EtOAc–Hex]; IR (thin film) 3400–3100 m, 1675 s; [^{1}H]NMR (acetone-d_6) 3.80 (s, 3H), 6.78 (t, J = 2.5 Hz, 1H), 6.93 (dd, J = 2.5 Hz, 1H), 8.69 (s, 1H), 9.84 (s, 1H); MS 153 (32), 152 (100), 151 (51), 123 (12), 108 (5), 69 (7).

4-[(tert-Butyldimethylsilyl)oxy]-2-hydroxybenzaldehyde ***(24).*** Using the same procedure as noted for the preparation of **17,** 5.0 g (36.2 mmol) of 2,4-dihydroxybenzaldehyde (Aldrich) was selectively silated over 18 hr to give 7.5 g (82%) of **24** as a clear oil: R_f 0.55 [20% (v/v) EtOAc–Hex]; IR (neat) 3700–2500 m, 1635 s; [^{1}H]NMR ($CDCl_3$) 0.24 (s, 6H), 0.97 (s, 9H), 6.37 (s, 1H), 6.44 (dd, J = 2, 9 Hz, 1H), 7.35 (d, J = 9 Hz, 1H), 9.68 (s, 1H), 11.37 (s, 1H); MS 252 (14), 197 (3), 196 (13), 195 (100), 75 (5).

4-[(tert-Butyldimethylsilyl)oxy]-2-methoxybenzaldehyde ***(25).*** To a stirred solution of 1.00 g (3.96 mmol) of **24** in 10.0 ml of CH_2Cl_2 was added 1.23 g (9.91 mmol) of Ag_2O, 2.38 g (19.8 mmol) of anhyd. $MgSO_4$, and 2.74 g (1.20 ml, 19.8 mmol) of CH_3I. The reaction vial was sealed and stirred in the dark at room temperature for 72 hr. After diluting with CH_2Cl_2 (20 ml) and suction filtering through a pad of diatomaceous earth, the combined organics were washed with 10% NaOH (2 × 10 ml) and brine (10 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by MPLC on a 1 × 30-cm column using 10% (v/v) EtOAc–Hex gave 0.79 g (75%) of **25** as a white, amorphous solid: mp 57–59°C; R_f 0.30 [35% (v/v) EtOAc–Hex]; IR (thin film) 1683 s; [^{1}H]NMR ($CDCl_3$) 0.26 (s, 6H), 0.98 (s, 9H), 3.86 (s, 3H), 6.44 (dd, J = 2, 9 Hz, 1H), 6.39 (s, 1H), 7.72 (d, J = 9 Hz, 1H), 10.29 (s, 1H); MS 266 (42), 210 (11), 209 (100), 181 (6).

4-Hydroxy-2-methoxybenzaldehyde ***(26).*** A solution of 2.6 g (9.8 mmol) of **25,** 6 ml of water, and 3 ml of acetic acid in 30 ml of THF was stirred at room temperature for four days. The solution was washed with water (10 ml), sat. aq. $NaHCO_3$ (3 × 10 ml), and brine (10 ml). The organic phase was concentrated in vacuo to give an off-white solid. Recrystallization from toluene (50 ml) gave 1.2 g (81%) of **26** as off-white needles: mp 158.5–159.5°C; lit 153°C (DeKiewiet and Stephen, 1931); R_f 0.11 [35% (v/v) EtOAc–Hex]; IR (thin film) 3400–2800 s, 1659 s, 1636 s; [^{1}H]NMR ($CDCl_3$/acetone-d_6) 3.87 (s, 3H), 6.43–6.60 (m, 2H), 7.68 (d, J = 9 Hz, 1H), 9.10 (br s, 1H), 10.22 (s, 1H); MS 152 (100), 151 (75), 135 (21), 121 (12), 108 (12), 92 (15), 39 (12).

5-Hydroxy-2-methoxybenzaldehyde ***(27).*** Using the procedure of Ulrich et al. (1974), 5.0 g (30.1 mmol) of 2,5-dimethoxybenzaldehyde (Aldrich) was selectively demethylated to give 1.4 g (31%) of **27** as a lemon-yellow solid: mp 111–112°C; lit 114–116°C (Ulrich et al., 1974); R_f 0.33 [35% (v/v) EtOAc–Hex]; IR (thin film) 3400–3000 m, 1658 s, 8383 s, 824 s; [^{1}H]NMR ($CDCl_3$–acetone-d_6) 3.86 (s, 3H), 6.81–7.30 (m, 3H), 8.00 (br s, 1H), 10.38 (s, 1H); MS 152 (100), 151 (27), 137 (25), 109 (28), 106 (29), 92 (36), 81 (50), 53 (49).

2-Hydroxybenzoic Acid, Ethyl Ester ***(28).*** A stirred solution of 2.0 g (14.5 mmol) of 2-hydroxybenzoic acid (MCB), 2.4 g (29.0 mmol) of anhyd. K_2CO_3, and 2.7 g (1.4 ml, 17.4 mmol) of iodoethane in 20 ml of acetone was refluxed for 8 hr. The reaction mixture was concentrated in vacuo, diluted with water (30 ml), and extracted with ether (3 × 15 ml). The combined extracts were washed with water (10 ml) and sat. aq. $NaHCO_3$ (2 × 10 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by flash chromatography on a 2.5 × 10-cm column using 20% (v/v) EtOAc–Hex gave 1.7 g (71%) of **28** as a clear oil: lit bp 226–228°C (Gottig, 1876); R_f 0.57 [35% (v/v) EtOAc–Hex]; IR (neat) 3500–2800 m, 1663 s; [^{1}H]NMR ($CDCl_3$) 1.37 (t, J = 7.5 Hz, 3H), 4.35 (q, J = 7.5 Hz, 2H), 6.69–7.10 (m, 2H), 7.24–7.58 (m, 1H), 7.81 (dd, J = 2, 7.5 Hz, 1H), 10.85 (s, 1H); MS 166 (17), 121 (13), 120 (100, 92 (11), 65 (4), 93 (3), 64 (3).

2-Hydroxy-3-methylbenzoic Acid, Methyl Ester ***(29).*** Using a procedure similar to the one used in preparing **1,** 5.0 g (32.9 mmol) of 2-hydroxy-3-methylbenzoic acid (Aldrich) was methylated to give 5.2 g (95%) of **29** as a clear oil: lit bp 239°C (Claisen and Eisleb, 1913); R_f 0.60 [35% (v/v) EtOAc–Hex]; IR (neat) 3550–2800 m, 1670 s, 750 s; [^{1}H]NMR ($CDCl_3$) 2.22 (s, 3H), 2.86 (s, 3H), 6.65 (d, J = 8 Hz, 1H), 7.21 (d, J = 8 Hz, 1H), 7.60 (d, J = 8 Hz, 1H), 10.49 (s, 1H); MS 166 (47), 135 (26), 134 (100), 106 (94), 105 (19), 78 (20), 77 (23), 51 (14).

2-Hydroxy-4-methylbenzoic Acid, Methyl Ester ***(30).*** Using a procedure similar to the one used in preparing **1,** 5.0 g (32.9 mmol) of 2-hydroxy-4-methylbenzoic acid (Aldrich) was methylated to give 5.1 g of **30** as a clear oil: lit bp 236–237°C (Biedermann and Pike, 1873); R_f 0.60 [35% (v/v) EtOAc–Hex]; IR (neat) 3450–2700 m, 1665 s, 770 s; [^{1}H]NMR ($CDCl_3$) 2.30 (s, 3H), 2.78 (s, 3H), 6.65 (d, J = 9 Hz, 1H), 6.75 (s, 1H), 7.66 (d, J = 9 Hz, 1H), 10.71 (s, 1H); MS 166 (46), 135 (35), 134 (100), 106 (30), 105 (20), 78 (29), 77 (24), 51 (15), 43 (15).

2-Hydroxy-5-methylbenzoic Acid, Methyl Ester ***(31).*** Using a procedure similar to the one used in preparing **1,** 2.0 g (13.1 mmol) of 2-hydroxy-5-methylbenzoic acid (Aldrich) was methylated to give 2.1 g (100%) of **31** as a light yellow oil: lit mp 2.5°C, bp 83–84°C (3 torr) (Carpenter and Easter, 1955); R_f 0.59 [35% (v/v) EtOAc–Hex]; IR (neat) 3500–2800 m, 1670 s, 820

s, 790 s, 765 s; [^{1}H]NMR ($CDCl_3$) 2.22 (s, 3H), 3.88 (s, 3H), 6.79 (d, J = 8.5 Hz, 1H), 7.16 (d, J = 8.5 Hz, 1H), 7.53 (s, 1H), 10.49 (s, 1H); MS 166 (39), 135 (27), 134 (100), 106 (26), 105 (17), 78 (26), 77 (23), 51 (16).

1-(2-Hydroxy-3-methylphenyl)ethanone ***(32).*** Using a procedure similar to the one used in preparing **4,** 3.0 g (19.7 mmol) of 2-hydroxy-3-methylbenzoic acid (Aldrich) was reacted to give 2.0 g (68%) of **32** as a light yellow oil: lit bp 103–104°C (9 torr) (Auwers et al., 1925); R_f 0.76 [35% (v/v) EtOAc–Hex]; IR (neat) 3500–2400 m, 1620 s; [^{1}H]NMR ($CDCl_3$) 2.23 (s, 3H), 2.59 (s, 3H), 6.75 (dd, J = 8 Hz, 1H), 7.30 (d, J = 8 Hz, 1H), 7.55 (d, J = 8 Hz, 1H), 12.54 (s, 1H); MS 150 (52), 136 (11), 135 (100), 79 (10), 77 (15), 43 (11).

1-(2-Hydroxy-4-methylphenyl)ethanone ***(33).*** Using a procedure similar to the one used in preparing **4,** 3.0 g (19.7 mmol) of 2-hydroxy-4-methylbenzoic acid (Aldrich) was reacted to give 2.5 g (84%) of **33** as a light yellow oil: lit bp 82–84°C (0.6 torr) (Julia and Chastrette, 1962); R_f 0.77 [35% (v/v) EtOAc–Hex]; IR (neat) 3480–2500 m, 1625 s; [^{1}H]NMR ($CDCl_3$) 2.30 (s, 3H), 2.54 (s, 3H), 6.61 (d, J = 8 Hz, 1H), 6.76 (s, 1H), 7.52 (d, J = 8 Hz, 1H) 12.27 (s, 1H); MS 150 (43), 135 (100), 107 (12), 77 (19), 43 (19).

1-(2-Hydroxy-5-methylphenyl)ethanone ***(34).*** Using a procedure similar to the one used in preparing **4,** 2.0 g (13.1 mmol) of 2-hydroxy-5-methylbenzoic acid (Aldrich) was reacted to give 2.0 g (100%) of **34** as a light yellow solid: mp 44.5–46.0°C; lit 50°C (Rosenmund and Schnurr, 1928); R_f 0.68 [35% (v/v) EtOAc–Hex]; IR (mineral oil) 3500–2500 m, 1640 s; [^{1}H]NMR ($CDCl_3$) 2.28 (s, 3H), 2.56 (s, 3H), 6.81 (d, J = 8.5 Hz, 1H), 7.24 (dd, J = 2, 8.5 Hz, 1H), 7.44 (d, J = 2 Hz, 1H), 12.10 (s, 1H); MS 150 (48), 135 (100), 107 (14), 77 (17), 43 (15).

1-(2-Acetyloxy-6-methylphenyl)ethanone ***(35).*** To a stirred solution of 1.28 g (8.5 mmol) of **4** in 5 ml of acetic anhydride at 50°C was added five drops of H_2SO_4. The reaction mixture was stirred 1.5 hr, cooled to room temperature, and diluted with ether (30 ml). The combined extracts were washed with sat. aq. $NaHCO_3$ (3 × 10 ml) and brine (10 ml), dried over $MgSO_4$, and concentrated in vacuo. Purification by flash chromatography on a 1 × 20-cm column using 10% (v/v) EtOAc–Hex, followed by MPLC on a 2.5 × 50-cm column using 10% (v/v) EtOAc–Hex gave 1.24 g (76%) of **35** as a light yellow oil: lit reported as an oil (Jones et al., 1981); R_f 0.33 [20% (v/v) EtOAc–Hex]; IR (neat) 1768 s, 1698 s; [^{1}H]NMR ($CDCl_3$) 2.22 (s, 3H), 2.28 (s, 3H), 2.42 (s, 3H), 6.82–7.37 (m, 3H); MS 192 (5), 50 (17), 136 (4), 135 (100), 77 (4).

RESULTS

Epiphytes. Several trends were apparent from the seed compounds isolated from individual species. First, very few of the epiphyte seeds contained monoterpenes (Table 2). Second, five aromatic compounds constituted the majority

of the compounds present in most of the samples (Table 1). The five components are: 2-hydroxy-6-methylbenzoic acid, methyl ester **1** also known as methyl 6-methylsalicylate (6-MMS); benzothiazole **2;** 4-hydroxy-3-methoxybenzaldehyde (vanillin) **3;** 1-(2-hydroxy-6-methylphenyl)ethanone **4;** and 1-(2,4-dihydroxyphenyl)ethanone **5** (Figure 1). These five prevalent compounds are likely the key compounds involved in any chemical cueing of the ants. Third, miscellaneous aromatic compounds exist in some of the epiphyte seed samples, but occur sporadically and without apparent pattern (Table 3).

The aromatic seed compounds are also present in the fruit juice and pulp and inner seeds and are probably localized in the seed coats. In *S. longifolius*, *E. phyllanthus*, and *M. ulei*, the fruit pulp and juice extracts contained significant quantities of all the prevalent aromatics found in the respective seeds (compare entries 1 and 3, 4 and 5, and 6 and 8 of Table 4). The extracts of *S. longifolius* seeds also contained large quantities of aromatics, even after washing the seeds repeatedly with pentane before grinding and extracting. This suggests that the seed coats house significant quantities of these compounds (compare entries 1, 2, and 3 of Table 4). Furthermore, the secondary extracts of *M. ulei* seeds still contained trace quantities of the prevalent aromatics when whole seeds were extracted for four days before grinding and reextracting with fresh pentane. Thus, the seed components can be housed inside the seeds or very deeply in the outer coat (compare entries 6 and 7 of Table 4).

Ants. GLC-MS analysis of the dichloromethane extract of the 350 whole heads of male *Ca. femoratus* ants revealed six minor peaks (3% or less), of which five had short retention times (<12 min), a major peak (81.2% of the sample) with a retention time of 14.5 min, and a peak (5.5% of the sample) with a retention time of 23.9 min. The mass spectra of the minor peaks were typical of aliphatics. The GLC-MS data also indicated that a trace ($\simeq 0.1\%$) of **1** was present (confirmed by coinjection).

The major peak at 14.5 min had a weak *m/z* of 156 and a fragmentation pattern typical of citronellol **6.** [^{1}H]NMR (compared with an authentic sample from Aldrich Chemical Co.), ORD, and CD spectra confirmed the presence of (−)-**6.**

(−)-Citronellol ***6.*** [^{1}H]NMR ($CDCl_3$) 0.92 (d, J = 6.6 Hz, 3 H), 1.15–1.25 (m, 1H), 1.31–1.43 (m, 2H), 1.55–1.67 (m, 2h), 1.62 (s, 3h), 1.69 (s, 3H), 1.95 (s, 1H), 1.97–2.04 (m, 2H), 3.63–3.74 (m, 2H), 5.09–5.14 (m, 1H); MS 156 (30), 123 (27), 119 (21), 95 (54, 82 (76), 81 (72), 71 (53), 70 (34), 69 (100), 68 (53), 67 (73); ORD (−) maximum at $\simeq$280 nm; CD (−) maximum at $\simeq$287 nm.

The peak at 23.9 min had a fragmentation pattern identical to that of mellein **7** (Brand et al., 1973b). In addition, the [^{1}H]NMR spectrum was identical to the published spectrum (Brand et al., 1973b). The ORD and CD spectra (Arakawa et al., 1969) obtained on this sample gave negative curves, indicating that (−)-mellein **7** had been isolated.

(−)-Mellein **7.** [^{1}H]NMR ($CDCl_3$) 2.95 (d, J = 7.2 Hz, 2H), 4.76 (six lines, spaced ≃6.7 Hz, 1H), 6.72 (dd, J = 1.2, 7.4 Hz, 1H), 6.91 (br d, J = 8.0 Hz, 1H), 11.05 (s, 1H), with the methyl group doublet reported to occur at δ 1.53 (Brand et al., 1973b) being obscured by impurities; MS 178 (100), 160 (46), 152 (17), 149 (23), 137 (39), 134 (86), 106 (14), 104 (14), 78 (16), 77 (17), 51 (15), 44 (16), 43 (37); ORD (−) maximum at ≃265 nm; CD (−) maximum at ≃255 nm.

Analysis of the sample of **7** at 446 nm indicated the presence of ≃5.7 μg of isolated material [observed absorption 0.116 with the sample in 2 ml of $CDCl_3$ ε ≃ 7200 (Patterson et al., 1966)]. Assuming equal percentage of isolation and equal GLC detector responses, then based on the GLC studies and the UV quantification of (−)-**7**, approximately 84.8 μg of **6** was isolated.

Analysis of GLC-MS data and coinjection studies on the oil obtained by pentane extraction of the ground heads indicated that **1** (2.1%), **6** (29.5%), and **7** (11.2%) were present.

The coinjection studies on extracts from the thorax and gaster indicated only compounds with high boiling points and retention volumes out of the range of the previously analyzed ant and epiphyte seed extracts. A trace of **6** was found (<5%), but could have resulted from contamination of the sample by components in the heads during decapitation.

DISCUSSION

The most likely candidate for a chemical cueing factor is **1**, as it is present in all the samples analyzed except the *F. paraensis* sample from Cocha Cashu (Table 1). Additional samples of *F. paraensis* must be analyzed to establish the absence of **1** as a general trend in this species. The other four prevalent aromatic compounds were not present consistently in all of the samples analyzed (Table 3) but may have an effect on the seed preferences demonstrated by the ants.

The discovery of identical seed compounds across taxonomically unrelated ant-garden epiphytes strongly suggests that these compounds could play some role in the ant-garden symbiosis. At least two possible categories of function are not mutually exclusive: (1) Seed compounds may attract *Ca. femoratus* workers, which then transport the seeds to the nutrient-rich nest substrate. (2) The seed chemicals could mediate interactions of the epiphytes and/or ants with other species in the forest environment. Although we cannot turn back the evolutionary clock to examine the origin of the symbiosis, we can review some circumstantial evidence supporting each of several scenarios.

First, evidence of **1** in both epiphyte seeds and *Camponotus* mandibular glands is compatible with Ule's (1906) hypothesis that seeds of ant-garden epiphytes attract ants by mimicking ant brood. Ule's theory was based on physical

similarities between seeds and brood, but ants are extraordinarily sensitive chemists, unlikely to be deceived by morphological mimicry.

Compound **1** (as well as **6** and **7**) is presumably produced in the heads of male *Ca. femoratus*, most likely in the mandibular glands, the major exocrine glands of ant heads. In males of other *Camponotus* species (Brand et al., 1973a,b; Payne et al., 1975; Blum et al., 1987), **1** is the principal exocrine product present in the mandibular glands. In *Camponotus herculeanus*, male mandibular gland products stimulate females to emerge from their nests to mate (Hölldobler and Maschwitz, 1965). Conservative tests indicate that *Ca. femoratus* does not sequester dietary **1** (Seidel, 1988), but had sequestration been possible in an ancestral ant-garden ant, sexual selection for enhanced male mating success could have stimulated the attraction of workers to **1.** Concentrations of **1** in male mandibular glands are markedly lower in *Ca. femoratus* than in closely related ants of the same subgenus (Brand et al., 1973a,b; Payne et al., 1975; Blum et al., 1987). This compound could have gradually lost its original function if its communicative value diminished in an environment saturated with the compound. Moreover, its function might have become altered, since changes in mating systems often or usually accompany the evolution of polygyny. Finally, although **6** had not been isolated previously from *Camponotus* ants, it has been found to occur in species of *Atta* (Myrmicinae) (Blum et al., 1968a), *Lasius* (Formicidae) (Law et al., 1965; Blum et al., 1968b), and *Acanthonyops* (Formicidae) (Regnier and Wilson, 1968) ants. In these species, **6** is present in the mandibular glands, but its function is unclear. It is tempting to speculate that in *Ca. femoratus*, **6** may have taken over some prior function of **1.**

Second, common seed compounds could mediate ant–plant interactions indirectly through their influence on other species. For example, an earlier experiment (Davidson, 1988) suggested that these chemicals repel ants that may be natural enemies of the plants (e.g., because they would disperse seeds to inappropriate microhabitats). Yet another possibility is suggested by the known fungistatic activities of seed compounds and their structural relatives. Both **3** and **5** are known fungistats (Fujikawa et al., 1952; Leifertova et al., 1975; Kurita et al., 1981), while derivatives and structural isomers of **1, 2,** and **4** also have fungistatic activity (Davies and Sexton, 1946; Sen and Joshi, 1952; Hayakawa et al., 1977). All have structural characteristics known to convey fungistatic activity (Greathouse and Rigler, 1940; Sen and Joshi, 1952; Leifertova et al., 1975). As suggested for bees that line their nest cavities with plant resins (Messer, 1985), ant-garden ants may rely on fungistatic plant compounds to retard growth of microbial nest pathogens. Although *Camponotus* ants lack metapleural glands, which are typical of most other ant genera (Hölldobler and Engle-Siegel, 1984), and often produce antiseptic secretions (Schildknecht and Koob, 1970, 1971; Maschwitz, 1974; Attygalle et al., 1989), ant gardens of

Ca. femoratus are not conspicuously riddled with fungal hyphae. Workers of this and related ant species possess powerful and volatile fungistatic mandibular gland products (Seidel, 1988; Blum, personal communication), but appear to use these secretions principally outside the nest (Davidson, 1988). *Crematogaster* species have metapleural glands, but ant gardens can grow successfully without resident colonies of *Cr.* cf. *limata parabiotica* (Davidson, 1988). Interestingly, mellein is both a metapleural gland product of at least one *Crematogaster* species (Attygalle et al., 1989) and a mandibular gland product of *Camponotus* (Brand et al., 1973a,b; Blum et al., 1987).

While the origins of symbiotic ant-garden relationships are not yet clear, preadaptions of plants and ants could have predisposed these groups for their associations with one another. Similarities in the seed compounds of ant-garden epiphytes could have arisen first as attractants for other kinds of seed dispersal agents. Vertebrates take the fruits of many or most ant-garden epiphytes and may be effective long-distance dispersal agents for these plants. However, because different ant-garden species attract different kinds of vertebrates (bats, birds, and/or monkeys), no single dispersal syndrome is an obvious potential predecessor to seed dispersal by ants (Davidson, 1988).

Another scenario of preadaptation appears more likely. Fungistatic seed compounds could have evolved prior to regular affiliation of ants and epiphytes, if these compounds contributed significantly to successful establishment in fungus-laden humus. Congeneric relatives of many ant-garden epiphytes are humus-dwelling taxa, whose seeds might have been exposed frequently to fungal infection in the decaying substrate where they germinated. Cloud forest relatives of ant-garden epiphytes regularly grow together without ants in the litter accumulating in epiphytic bromeliads. At lower elevations, ants may originally have occupied similar sites for the shelter and protection these microhabitats afforded (Davidson and Epstein, 1989). Whether nesting in bromeliad litter or in arboreal carton nests lacking epiphytes, ants could have retrieved seeds initially because of their chemical similarity to brood, or simply as a means of carrying nutritious fruit pulp. Feeding by ants on sweet fruit pulp is perhaps a natural evolutionary consequence of frequent contact with material that is nutritionally similar to homopteran exudates.

The possibility that plants and ants were preadapted for their present-day interaction does not preclude the potential for evolutionary specialization or coevolution following the initial establishment of their associations. Regular seed dispersal to ant nests should have imposed strong and consistent selective regimes across both epiphyte and ant life histories, including critical stages of early establishment. Plant growth rates could have increased in response to richer nutrient medium, and escalated ant aggression and polygyny might be evolutionary responses to increasing availability of ant resources. Higher concentrations of seed compounds could have been selected for if they enhanced dispersal to favorable microsites, or increased resistance to microbial pathogens in organic

substrate now supplemented with feces of ants and vertebrates. Tests of these scenarios for special adaptation would require comparisons between ant-garden species and their closest relatives, but accurate phylogenies are not yet available for either epiphyte or ant taxa. At present, no direct evidence supports the hypothesis of reciprocal coevolution between ant-garden ants and their epiphytes (Davidson and Epstein, 1989).

Whether seed compounds evolved before or after epiphytes became associated with ants, the presence of compounds **1** and **2** in so many unrelated taxa may be a spectacular case of convergent evolution. However, we cannot yet rule out two alternative hypotheses. First, plants might have evolved their compounds with respect to unrelated selective pressures but become concentrated in a single environment by ants responding selectively to particular seed compounds. Second, and perhaps most parsimonious for long-lived colonies of social insects, the capacity to produce seed compounds could be transmitted "culturally." Thus, ants might infect their cultivated epiphytes with symbiotic microflora, conferring the very property that encourages seed dispersal and future cultivation.

Finally, for ant-gardens, as for human agricultural systems, it is interesting to speculate as to whether the animals have "domesticated" their plants or vice versa (Davidson and Epstein, 1989). Aggressive ants tending homoptera on fruiting pedicels could have "captured" plants by deterring vertebrate dispersal agents. Alternatively, Ule's brood mimicry hypothesis suggests that epiphytes (especially those whose seeds lack nutritional appendages) could have evolved the capacity to deceive ants and manipulate their behaviors. Although social parasites of ant colonies have long been known to manipulate ant behavior with nonnutritional chemical cues (reviewed in Kistner, 1979), ours are the first data suggesting that myrmecophytic plants may do the same.

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OVIPOSITIONAL RESPONSE OF THREE *Heliothis* SPECIES (LEPIDOPTERA: NOCTUIDAE) TO ALLELOCHEMICALS FROM CULTIVATED AND WILD HOST PLANTS[1]

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Abstract—The role of plant allelochemicals on the oviposition behavior of *Heliothis virescens* (F.), *H. subflexa* (Guenee), and *H. zea* (Boddie) was investigated in the laboratory using a "choice" bioassay system. Fresh young leaves of tobacco, *Desmodium tortuosum* (Swartz) de Candolle, groundcherry (*Physalis angulata* L.), and cotton (*Gossypium hirsutum* L.) squares (flower buds) were washed in methylene chloride or methanol, concentrated to 1 g equivalent of washed material, and applied to a cloth oviposition substrate. Each of the extracts—including groundcherry, a nonhost—stimulated oviposition by *H. virescens*. *H. subflexa* were stimulated to oviposit by groundcherry extract, its normal host, and extract from cotton squares, a nonhost. None of the extracts stimulated oviposition by *H. zea*, although all except groundcherry were from reported hosts. The sensitivity of the bioassay was confirmed by giving *H. virescens* and *H. subflexa* an opportunity to choose between extracts that showed stimulant qualities when tested independently versus only solvent-treated controls. In these tests, tobacco showed the highest level of stimulant activity for *H. virescens*; groundcherry exhibited the highest level of stimulation for *H. subflexa*.

Key Words—Cotton, groundcherry, tobacco, *Gossypium*, *Desmodium*, *Physalis*, plant–insect interaction, host-plant resistance, *Heliothis* spp., Lepidoptera, Noctuidae, oviposition stimulant, oviposition deterrent.

[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or the recommendation of its use by USDA.

INTRODUCTION

Incorporation of factors into cultivated plant species that reduce or eliminate insect pest populations is an efficient and economical approach to the management of crop insect pests. Although there have been some dramatic demonstrations of the effects of plant resistance on the management of insect pests, progress has been extremely slow in some crops because of the empirical selection process that often requires many years of observation and experimentation before a cultivar can be released to growers. It now is generally believed that chemicals are the basis for many plants' defense against insect attack and that the chemical defense systems are composed of not one but a spectrum of chemical compounds (Alborn, 1988, and references therein). Although plant breeders have been successful in producing single-gene resistant material, little is known about plant chemicals imparting resistance in plants. To become more specific in breeding efforts, breeders must know how the behavior and biology of the insect pest is influenced by the plant's defense system, and how it can be modified to obtain an increased and more durable resistance.

Heliothis spp. rank high among the most destructive crop pests in the United States and around the world. Mated females of *Heliothis* spp. typically deposit their eggs on favored host plants (Johnson et al., 1975; Roome, 1975). Upon hatching, the feeding larvae often inflict extensive damage to flowers and fruit, resulting in millions of dollars in crop losses and control costs. The basis for the females of *Heliothis* spp. choosing one plant species or cultivar over another is poorly understood. Recently, Jackson et al. (1984a) showed that cuticular components from green leaves of a typical flue-cured tobacco (NC 2326), *Nicotiana tabacum* L., stimulates oviposition by *H. virescens* (F.) (Hv). Further, in comparative choice tests in oviposition cages, Hv females consistently oviposited fewer eggs on a primitive tobacco introduction, TI 1112, than on NC 2326 (Jackson et al., 1983). The primitive tobacco's "resistance" to oviposition by Hv is due, at least in part, to the absence or greatly reduced level of oviposition stimulant compounds present in the cuticular washes from the oviposition "susceptible" tobacco, NC 2326.

Johnson et al. (1975) reported on the ovipositional response of *H. zea* (Boddie) (Hz) to different phenological states of its major host plants in North Carolina. They showed that the flowering periods are the most preferred phenological states for corn, cotton, tobacco, and soybean, although Hz and other *Heliothis* spp. will oviposit on certain host plants in the absence of fruiting or flowering structures. Roome (1975) reached similar conclusions concerning the oviposition behavior of *H. armigera* (Hübner) (Ha) in corn and sorghum. He also suggested that susceptible crops attract adults and that, once within the crop, mated females are "trapped" by suitable physiological cues from the plants.

Identification of the chemical cues from plants that affect oviposition could assist in the breeding of crop cultivars resistant to *Heliothis* attack. The present study was conducted to determine the effect of cuticular washes from host and nonhost plants on the oviposition behavior of Hv, Hz, and *H. subflexa* (Guenee) (Hs). Unlike the polyphagous Hz, Ha, and Hv that attack a wide variety of cultivated crops, Hs is a monophagous species feeding exclusively on groundcherry, *Physalis* spp. (Brazzel et al., 1953). Groundcherry generally is regarded as a weed in the United States, but a commercial cultivar of "tomatillos" (*Physalis ixocarpa* Brot.) is widely grown in Mexico (Saray and Loya-Ramirez, 1978).

METHODS AND MATERIALS

Bioassay. The lack of suitable analytical chemical methods and appropriate bioassay techniques have heretofore hindered the study of insect–plant interactions relative to host finding and oviposition behavior. The methodology traditionally used in the study of insect–plant chemical relationships generally requires large quantities of plant material. For these bioassays, a recently developed olfactometer (Mitchell and Heath, 1987) was used that permits study of the effects of plant allelochemicals on insect oviposition behavior throughout the year in a controlled environment.

The bioassay chambers were located in an environmentally controlled room 3 m long, 2.6 m wide, and 2.1 m high. The room was equipped with an electric timer to turn overhead fluorescent lights (two banks of two bulbs, 40 W each) on and off; a separate timer was used to turn the red lights over the individual chambers on and off. A heat pump–air conditioner controlled room temperature at ca. 28.6°C. A room humidifier maintained the relative humidity at ca. 50%.

Test Insects. All test insects were reared in the laboratory on a modified pinto bean diet using methods described by Guy et al. (1985), King and Moore (1985), and Mitchell et al. (1988). The pupae of each of the three species—Hv, Hz, and Hs—were sexed and held separately in 3.8-liter paper cartons with screened tops until emergence. Upon emergence, females and males (21 : 14) were confined together for mating in 3.8-liter cartons for two days. Test insects were not routinely sacrificed to check mating status. However, periodic checks were made on the mating of sacrificed moths, and >90 were found to be mated as evidenced by the presence of a spermatophore in the bursa copulatrix. The pupal emergence and mating cages were held under a reverse 14 : 10-hr light–dark cycle in holding rooms maintained at ca. 28°C and 50–60% relative humidity.

Plant Material. One hundred grams of fresh cotton squares (i.e., flower buds), whole leaves of cotton (variety McNair 220), tobacco (susceptible, NC

2326; resistant, TI 1112), groundcherry (*Physalis angulata* L.), and *Desmodium tortuosum* (Swartz) de Candolle were washed in 500 ml solvent for 30 sec. *D. tortuosum* is an important late-season weed host for Hv and Hz in the Florida–Georgia tobacco belt (Jackson et al., 1984b; Jackson and Mitchell, 1984). The whole leaf wash was filtered through a white Viva paper towel into 1-liter containers, which were then capped and stored at 10°C until needed (1–6 months) (Mitchell and Heath, 1987).

The tobacco plants used were in the prebloom stage; the cotton, groundcherry, and *Desmodium* plants were in the flowering stage. All plant material was grown outdoors under natural light in small field plots. The leaves selected for washing were fully expanded yet tender and located on the upper half of the plants near flowering points. All plant materials except groundcherry were washed in methylene chloride; groundcherry was washed with methanol. Preliminary trials with whole-leaf washes showed that methanol was ineffective for extracting stimulant materials from cotton, tobacco, and *D. tortuosum*. Similarly, methylene chloride was ineffective for extracting oviposition stimulant compounds from groundcherry leaves (Mitchell and Heath, 1987).

Test Procedure. For testing, the plant extracts were concentrated in a rotary evaporator. A 1-g equivalent of whole leaves or squares in 1 ml of solvent was pipetted in a circular pattern onto the center of a piece of white broadcloth, the oviposition substrate (Mitchell and Heath, 1987). Broadcloth treated with 1 ml solvent only was used as the control. New cloths were used in every test. In preliminary tests, cloths treated with methylene chloride or methanol had no effect on egg deposition when compared to cloths without these solvents. After drying, the cloths were fitted over the open end of opposing collars and secured in position with a plastic O-ring. The other two openings in the chamber top were plugged with clear plastic cups. A cotton ball soaked with 10 ml water was then placed on the top of each cloth over the treated area and covered with a paper cup. Pretest measurements showed that the paper cup had no significant effect on air flow.

Each test chamber contained six Hv or Hs females or three Hz females. Fewer Hz females were used because of its more prolific egg-laying habit compared to Hv and Hs. The females were mated, as described, before being placed in the chambers. Periodic checks on the mating status of moths made throughout the course of the study showed that $>90\%$ of the females had mated after two days of confinement with males.

The females were preconditioned through one complete light–dark cycle in the test chambers before being exposed to the test materials; thus, the females were 3 days old when first subjected to treatment. Each test was usually repeated the following day (moths 4 days old) and sometimes on the third day (moths 5 days old), after which the females were removed from the test chambers. The position of the treatments in each compartment were alternated daily. The moths

were supplied daily with fresh food (10% honey–water solution on a saturated cotton ball in a paper cup placed in the chamber).

Choice tests also were conducted using either two plant extracts or three extracts and a control cloth in the same compartment. Dual-choice opportunities were presented to both Hv and Hs; the four-choice arrangement was presented only to Hv. Treatments in the dual-choice tests were alternated daily as described. Treatments involving four choices were randomly positioned in each compartment at the start of the test. The treatments then were rotated clockwise daily until each treatment (i.e., extract) was exposed once at each of the four ports in the test compartment. Freshly treated cloths were used daily in all tests.

The tests were carried out under a reverse photoperiod of 14 : 10 hr light–dark. Treatments were applied at 1200 hr. The test cloths were removed 4 hr later, and the eggs were counted. Treatment means were separated using the paired *t* test (Steel and Torrie, 1960) or Duncan's multiple-range test (Duncan, 1955).

RESULTS AND DISCUSSION

Each extract tested—including groundcherry, a nonhost—stimulated oviposition by Hv (Figure 1). Hs adults were stimulated to oviposit by groundcherry extract and cotton squares (Figure 2). The positive oviposition response of Hv to washes from known host plants (cotton, tobacco, and *D. tortuosum*) is consistent with their polyphagous habit.

Laster et al. (1982) reported on the host acceptance and development of Hs, Hv, their hybrid, and backcross progeny on several plant species. In their study, none of the Hv larvae placed on leaves of groundcherry survived. Similarly, none of the Hs larvae placed on cotton leaves survived (Laster et al., 1982). These results suggest that although these species possibly may be stimulated to oviposit on nonhosts under forced conditions, e.g., Hv on groundcherry and Hs on cotton leaves, it is unlikely that any resultant larvae would survive in nature.

Cloths treated with extract from groundcherry and cotton squares had significantly fewer Hz eggs compared to solvent-treated control cloths. The chemical basis for this deterrence is unknown. However, among other moth species it is not unusual for chemicals extracted from nonhost and host plants to exhibit varying levels of deterrence by ovipositing females when sprayed onto otherwise acceptable host plants (Tingle and Mitchell, 1984, 1986; Mitchell and Heath, 1985; Williams et al., 1986).

The sensitivity of the bioassay was further demonstrated by giving Hv an opportunity to choose to oviposit between extracts of susceptible tobacco (NC 2326) and resistant tobacco (TI 1112) (Jackson et al., 1984a). In our test, Hv

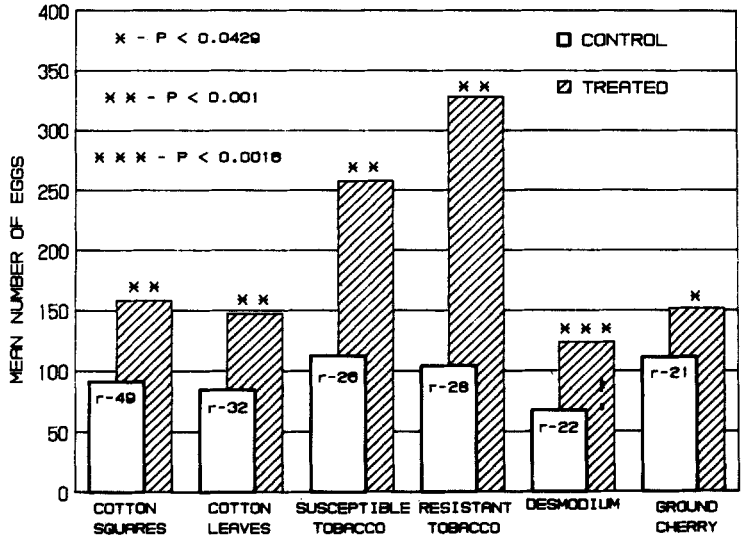

FIG. 1. Effect of plant extracts on the oviposition response of *Heliothis virescens* in laboratory bioassays. The *r* values represent the number of replications per test. Asterisks indicate the level of probability between means, paired *t* test, (Steel and Torrie 1960).

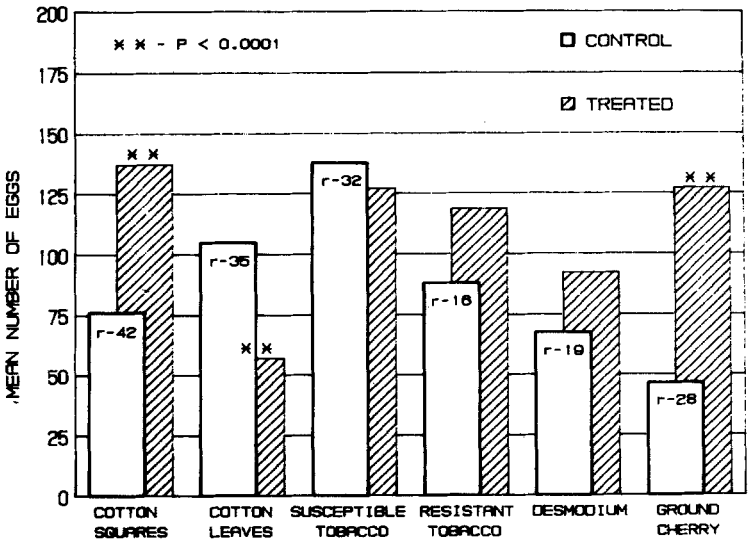

FIG. 2. Effect of washes from leaves and cotton squares (flower buds) on oviposition by *Heliothis subflexa*. The *r* values represent the number of replications per test. The asterisks indicate the level of probability between means; absence of asterisks indicates no significance between means, paired *t* test (Steel and Torrie, 1960).

laid more eggs on cloths treated with susceptible extract than on cloths treated with resistant tobacco extract (Figure 3). These results are consistent with those of Jackson et al. (1983), who conducted similar competitive tests outdoors in small field cages. It should be noted that the moths had free access to both treated and untreated cloths (this study) or treated and untreated plants (Jackson et al., 1983). Thus, the moths could either accept or reject each cloth or plant in a sequential manner. In oviposition tests such as these, it is the final egg numbers that are tallied and analyzed. These sorts of tests do not differentiate between olfactory and tactile stimuli. Moths may be attracted to a source over a distance—albeit small—but simply not oviposit on it. On the other hand, moths also may find a nonattractive substrate (cloth or plant) by accident but be highly stimulated to oviposit upon making contact.

In other dual-choice tests using susceptible tobacco (NC 2326) as the standard, there was no significant difference in the mean number of eggs deposited by Hv on cloths treated with tobacco extract or groundcherry extract (Figure 3). However, cloths treated with extract from cotton leaves received only ca. 50% of the number of eggs deposited on cloths treated with tobacco leaf extract. By contrast, cloths treated with *Desmodium* extract showed a slight but significant increase in the number of eggs deposited on them compared to cloths treated with tobacco extract (Figure 3).

Initial experiments with Hs suggested that cotton squares possessed ovi-

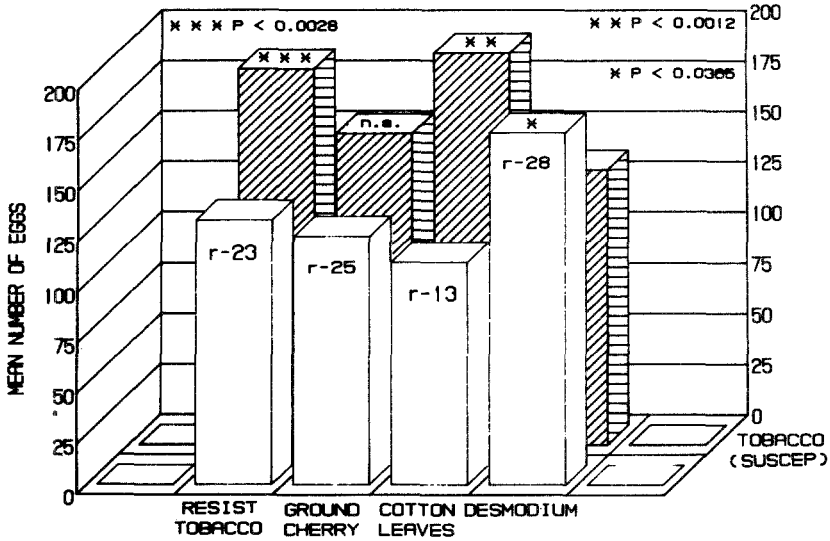

FIG. 3. Oviposition response of *Heliothis virescens* exposed simultaneously to two plant extracts. The *r* values represent the number of replications per test. Asterisks designate the level of significance between treatment means; n.s. indicates that there was no significant difference, paired *t* test (Steel and Torrie, 1960).

position stimulant qualities comparable to groundcherry, the only known host for this species (Figure 2). However, results of dual-choice tests with groundcherry and cotton squares or *Desmodium* clearly showed that this was not the case. In these two tests, cloths treated with groundcherry extract received ca. 70% of all the eggs deposited by Hs.

Results of the test in which Hv were exposed simultaneously to an untreated control and extracts from susceptible tobacco (NC 2326), *Desmodium*, and groundcherry are shown in Figure 4. When tested independently, each of the three extracts showed a significant increase in oviposition by Hv when compared to solvent-treated control cloths (Figure 1). The differences noted in egg deposition in the multiextract test were highly significant and consistent with the results of the tests involving a single treatment versus control cloths. The positive ovipositional response recorded from Hv from whole-leaf wash of susceptible tobacco, NC 2326, probably is due to the presence of duvane diterpenes secreted from leaf trichomes (Jackson et al., 1986). Conversely, the ovipositional nonpreference exhibited by Hv towards TI 1112 probably was due to the lack of or reduced level of duvane diterpene secretions.

It is possible that the stimulatory effects of the various extracts on egg-laying activity by Hv were caused by the same compounds or compounds similar to those found in tobacco, i.e., duvane diterpenes. A more realistic assump-

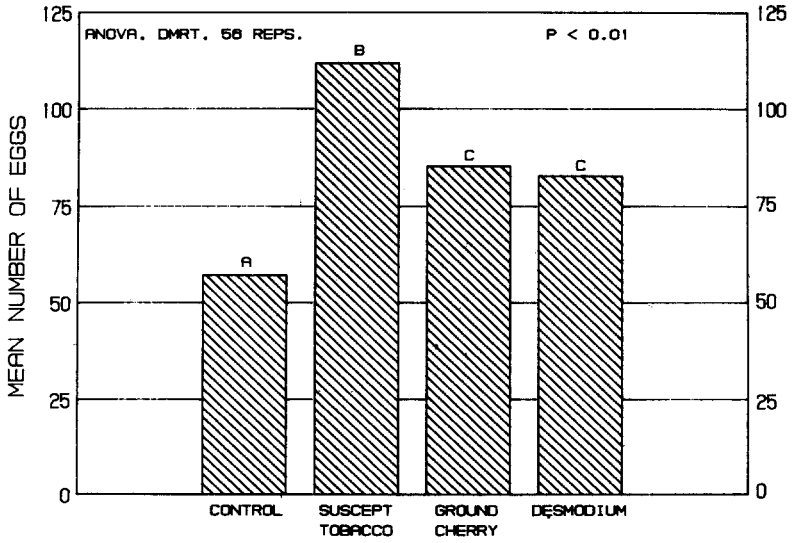

FIG. 4. Oviposition response of *Heliothis virescens* when exposed simultaneously to three plant extracts and a control. The *r* values represent the number of replications per test. Means with the same letter are not significantly different, Duncan's multiple-range test (Duncan, 1955).

tion, however, would appear to be that Hv are stimulated to oviposit by several different compounds. Such diversity in stimulant chemicals would have obvious advantages to an insect such as Hv, which is dependent for its survival upon many species of annual host plants.

The stimulatory oviposition response recorded for Hv from groundcherry extract (Figure 1) lends further support to the hypothesis that different species of plants may have different chemicals that elicit the same type of behavioral response, e.g., increased egg-laying. There is no evidence, however, that Hv oviposits on groundcherry plants in nature.

The lack of a stimulatory egg-laying response by Hz to any of the plant extracts was surprising, especially since all but groundcherry are known hosts. Johnson et al. (1975) reported on the ovipositional response of Hz to several crop hosts including tobacco and cotton. Comparing the flowering states of the crops, the maximum ovipositional response decreased in the following order: corn > tobacco > soybean > cotton. Their results clearly showed that Hz exhibited a strong ovipositional preference for flowering corn over tobacco, soybean and cotton.

Several researchers have cited chemical factors from corn silks as possible attractants or oviposition stimulants for Hz (Wiseman et al., 1988, and references therein). We did not test extracts of corn silk in our bioassay apparatus. Failure of our bioassay to demonstrate the presence of oviposition stimulating compounds for Hz in extracts from cotton and tobacco does not preclude their presence. Clearly, the work of Johnson et al. (1975) suggests that cotton and tobacco do have such compounds. All of the extracts used here were tested at only one dosage level—1 g equivalent of whole leaf or square wash. Thus, it is possible that the dosage used in these tests was below some as yet unknown critical threshold level for oviposition stimulant activity. It is also possible, of course, that Hz females simply did not respond "normally" in the confines of our bioassay apparatus.

Recent reports by Rembold and Tober (1985) showed that Ha females responded differentially in oviposition trials to odors emanating from two cultivars of pigeonpea, *Cajanus cajan* L. Millsp. Similarly, Tingle et al. (1989) found that Hs mated females exhibited positive flight responses to odors emanating from extracts of leaves of its host, groundcherry. Likewise, we have found that Hv females are attracted over a distance to volatile chemicals released from extracts from cotton, tobacco, and *Desmodium* (unpublished data).

Clearly oviposition per se is but one of a complex of behavioral events regulating the selection and successful colonization of host plants by insects. Nevertheless, identification of the chemicals stimulating oviposition in Hv presents opportunities for characterization of the behavioral and physiological factors regulating this essential process in the life cycle of this important pest. Further, it is conceivable that such chemicals may be greatly reduced or elim-

inated from otherwise desirable cultivars through genetic manipulations, thereby imparting a degree of "resistance" to attack by *H. virescens*.

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CHILDREN'S SENSITIVITY TO ODOR OF TRIMETHYLAMINE

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Abstract—Findings in this paper show a strong correlation between subjects' age and their olfactory sensitivity to the "fishy" odor of trimethylamine, with youngest subjects being most sensitive and adult subjects least sensitive to this odor. This was due to a high percentage of highly sensitive subjects in the youngest age groups; this percentage decreased with age. Data further support the notion that trimethylamine sensitivity is independent of sex. The sensitivity to trimethylamine per se showed no significant covariations with the subjects' preferences for or aversions against fish as food and is probably of minor importance for fish food acceptability.

Key Words—Odor sensitivity, trimethylamine, children.

INTRODUCTION

A recent survey of a representative sample of Norwegian households has shown that 19% of the included children strongly rejected fish dinners, while adult household members on the average were more willing to eat fish (Jellestad and Solbu, 1987). These results call for both psychological and physiological explanations.

Trimethylamine (TMA) might be one significant olfactory cause for fish food rejection. It has a typical "fishy" odor in low concentrations (Standsby, 1962) and is a substantial contributor to the smell of dead fish (Jones, 1967). TMA occurs in dead saltwater fish as a bacterial degradation product, increasing in concentration as fish muscle decomposes (Huss, 1983; Smith et al., 1980).

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In living fish TMA is found as odorless TMA oxide (TMA-O). TMA-O may represent as much as 7% of the dry weight of some fish species, depending on the season and location of catch (Huss, 1983, p. 18). Certain species of bacteria split TMA-O into TMA and oxygen by anaerobic respiration. During the degradation process, free TMA accumulates in the muscle tissues, enabling the fish industry and fish food control authorities to use TMA as an indicator of fish freshness and quality (Shewan et al., 1971; Castell et al., 1974; Ritskes, 1975).

The average threshold of adults to the smell of TMA is very low. Leonardos et al. (1969) has found the TMA recognition threshold to be 0.00021 parts TMA to one million parts of air; this is the lowest threshold of the 53 commercially important odorants in their study, including hydrogen sulfide and methyl mercaptan. Amoore and Forrester (1976) have reported similar results using a more easily available sniff-bottle method, the average TMA threshold for adults being 0.0005 ppm (0.0005 parts TMA to one million parts of water).

As far as we know, children's sensitivity to the odor of TMA has not been published previously. Children are often claimed to have a more "sensitive nose" than adults. This postulate is not satisfactorily documented. On the other hand, the sense of smell is weakened during adulthood and may be considerably deteriorated in the elderly (Murphy, 1987, p. 253). If this developmental tendency begins in early childhood, the sensitivity of children to TMA should be measurably higher than for adults when these two groups are tested under similar conditions. This also opens the possibility that children might detect TMA in lower concentrations than adults when TMA is a deterioration product in raw fish muscle or a gas set free during cooking.

With this background, a study of children's sensitivity to the smell of TMA was deemed useful as a possible physiological contributor to the cause of fish food rejection and a possible documentation of the impression that children in general are more odor-sensitive than are adults.

METHODS AND MATERIALS

Subjects. This study originally included 356 subjects. Five were excluded because of major data loss or errors; the final sample consisted of 322 pupils and 29 adults. The children's group consisted of 168 girls (52.3%) and 153 boys (47.7%), their ages ranging from 6 to 16 years, the average being 11.0 years (SD 3.1). The sex classification for one subject was lost. Pupils from nine schools were represented in the sample.

The adult group consisted of 15 teacher-training students, eight psychology students, and five employees at the Institute of Physiological Psychology; 19 were women (65.5%) and 10 were men (34.5%). The age of the adult group was 20–42 years with a mean age of 24.5 years (SD 5.3).

All 351 subjects were tested during daytime in their respective school or work environments. No subjects were paid for their participation in the study.

Equipment. Glass tubes with an inner diameter of 15 mm and length of 18 cm were used for the odor sensitivity study. These were filled with 10 ml of liquid, raising the liquid level to 11.5 cm from the tube rim. All tubes had odorless airtight screw-caps permitting odorants to be prepared some hours in advance.

Four tube-rack sets of odorants were used, each of these containing 40 tubes and being easily transportable. The use of four sets made simultaneous testing of four subjects possible. Twenty tubes in a set contained TMA diluted in a buffer solution controlling the pH value, the remaining 20 tubes contained buffer solution only. Tubes were arranged pairwise with one TMA tube and one buffer tube. The pairs were numbered from 18 to 37 corresponding with the step of dilution of the TMA in the TMA tube, and the tubes were also coded with either a V (left) or an H (right), depending on their position in the tube pair.

Commercially available crystalline trimethylamine hydrochloride (Sigma) was used in the experiment. Numbered polypropylene beakers were used for the dilution series, one for every dilution step.

Dilution Steps and Buffer. Buffer was made by dissolving 20.02 g $KHCO_3$ in 1000 ml distilled water. This solution was adjusted with 2 M KOH to pH 9.74, the 50% ionization point (pK_a) for TMA. Bicarbonate buffer then was diluted to 0.1 M by adding 1 liter of distilled water.

As a starting point for the dilution series, TMA hydrochloride saturated in buffer was used, representing dilution step 0. Purified TMA has a solubility of 410,000 ppm (w/v) in water (Freier, 1976; Amoore and Forrester, 1976), while the chemical literature gives contradictory information as to the solubility of TMA hydrochloride.

Fifty microliters of saturated solution were added to 204.8 ml buffer, corresponding to dilution step 12. Four milliliters of step 12 was pipetted into 28 ml buffer (step 15). Twenty milliliters of step 15 was added in 140 ml buffer (step 18). A 50% dilution procedure was then used down to step 37 by taking out 50 ml and diluting this in 50 ml buffer in a numbered beaker, sealed with laboratory film, and mixed. Ten milliliters were then pipetted into four tubes which were immediately capped. Then 50 ml was taken out of the beaker and added to 50 ml buffer in a new beaker; the procedure with sealing, mixing, and pipetting being repeated down to dilution step 37.

Procedure. At the most, four test administrators were collecting data simultaneously, one in each corner of the room in use. Each test administrator had one set of tubes and a flask with buffer solution. Subjects were first asked to smell the buffer solution (this allowed an olfactory reference and an exchange of gas in the nasal cavities) and were told to take "one good sniff" of each

tube (this was demonstrated). They also were told that the two tubes smelled different and were instructed to point out the tube in each pair with the stronger odor. If they did not recognize any difference, they should report that the tubes smelled the same.

Tube racks were arranged so that the TMA concentration in the TMA tube was doubled every time a new pair was presented to the subject. Tubes were uncapped, the caps being placed on left or right marked paper sheets to prevent exchanging the caps by accident. The placing of TMA tubes in relation to buffer tubes in the racks was taken from a published randomization table (Gellermann, 1933). When presenting the tubes, the test administrator held one tube in each hand, keeping them at a good distance from each other. Subjects first smelled the left tube, then the right. Tubes were kept at a 1- to 2-cm distance from beneath the subject's nose, and the subject sat with arms at rest when not pointing out the tubes. Subjects were not allowed to try more than once at each dilution step.

Establishing Sensitivity. The criteria for establishing the subjects' sensitivity to TMA was five correct choices of tubes in a row, the sensitivity measure referring to the first and most diluted TMA concentration of these five correct choices.

Because of the time factor and the subjects' limited attention span, we could not allow subjects to smell more tubes than necessary. A code on the scoring sheet enabled the test administrator to end the test when a sensitivity measure was attained. For this reason the procedure was not double-blind. A rack with only buffer tubes was used to control for the lack of a double-blind procedure. Neither test administrator nor subjects were informed about this control set in advance. Four of 34 subjects (11.8%) got a "sensitivity measure" established with this set, despite both parties' expectation of the tubes being odorous. The chance of meeting criteria by randomized choices was 3.1%.

Test Localities. The experiments were mostly carried out in classrooms. Test conditions were determined by tube temperature (20–22°C), room temperature (19–22.5°C), facilities for handwashing, and ventilation. No rooms were smaller than ordinary classroom size. A subjective background odor evaluation was made, resulting in two occasions when background odor was easily detected when first entering these rooms. Results from subjects tested in these rooms were statistically compared with results from other subjects in the same age groups, revealing no significant differences, allowing these subjects to be included in the data analyses.

Additional Data. Immediately before the TMA sensitivity measurements, subjects were given a questionnaire or a structured interview concerning their fish food preferences and aversions, as well as questions more directly related to the sensitivity experiment. This was done to study possible relations between sensitivity measures and food habits.

Data Analysis. In the statistical analyses, the programs *t* test, Mann-Whitney U test, ANOVA, Kruskal-Wallis ANOVA by ranks, and correlation from the statistical packet CSS (Complete Statistical System) (StatSoft Inc., 1987, 1988) were used.

RESULTS

Results are based on 322 pupils. Thirty-four subjects were tested with the control set, leaving 288 subjects in the TMA sensitivity test. Of these, 33 (11.5%) failed to get a sensitivity measure according to current criteria and were excluded from the calculations, leaving 255 subjects (88.5%) with established sensitivity measures.

Figure 1 presents the children's frequency distribution on the dilution series. The children's group as a whole got an average score of 25.5. Thirty-one children and one adult obtained a TMA sensitivity score of 31 or higher. These "highly sensitive" subjects affected the age groups' average scores to a different degree, as presented in Figure 2.

In Figure 2 the sensitivity to TMA is expressed in dilution steps. Estimation of the real TMA concentrations (ppm; w/v) in the dilution series was difficult because of contradictory findings in the chemical literature, and, for the sake of simplicity, only dilution step results are presented.

The youngest age group obtained an average score of 28.2: TMA halved

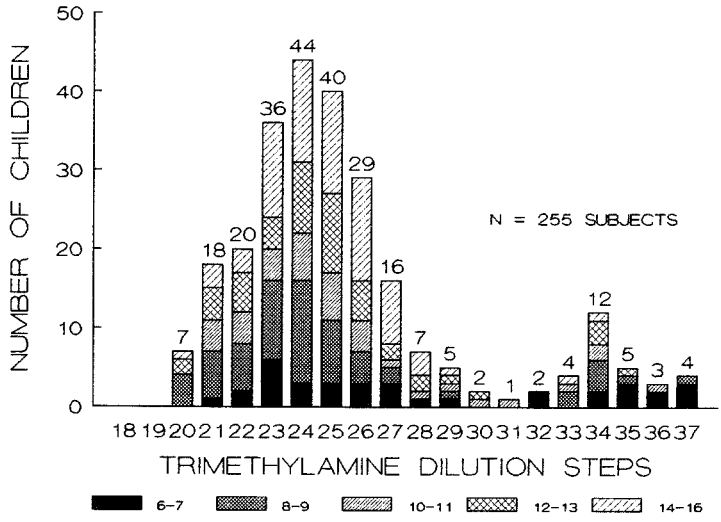

FIG. 1. Sensitivity of children to trimethylamine odor: frequency distribution.

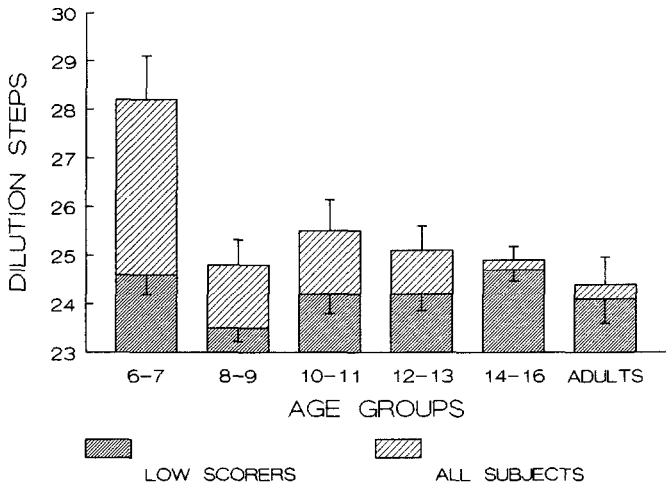

FIG. 2. Sensitivity of several age groups to trimethylamine odor: group means and standard error.

28.2 times. When subjects ages 6–7 with scores of 31–37 were taken out of the analysis, the average score dropped to 24.6, corresponding to the TMA saturated dilution halved 24.6 times.

The TMA sensitivity for the sample as a whole showed a significant negative correlation with age ($r = -0.16$, $P = 0.008$). A one-way analysis of variance showed a strong relation between age groups and the ability to smell TMA ($F = 5.40$, $P = 0.001$). Since rather large group standard deviations were found, a nonparametric Kruskal-Wallis analysis also was carried out. This supported the previously reported results ($H = 13.02$, $P = 0.01$).

The entire age group 6–7 years was significantly different from older pupils ($F = 20.99$, $P = 0.000$), and from the adult group ($F = 11.09$, $P = 0.002$). Children as a whole, however, were not significantly different from the adult group. Subjects ages 8–16 showed no statistically significant differences from adults in their sensitivity to TMA. With the high sensitivity group removed, no significant age differences were found exept for the subjects ages 8–9, who scored significantly lower than the 6- to 7-year-old group ($T = 2.3$, $P = 0.02$) and the 14- to 16-year-old group ($T = 3.6$, $P = 0.001$, two-tailed).

A closer view of the 32 subjects with scores 31–37 revealed a high percentage of "highly sensitives" in the 6- to 7-year-old group, the percentage decreasing with age. This is shown in Figure 3.

No significant sex differences were found concerning the subjects' ability to sense TMA. This is shown in Table 1 which also presents means, standard deviations, and standard errors for all age groups.

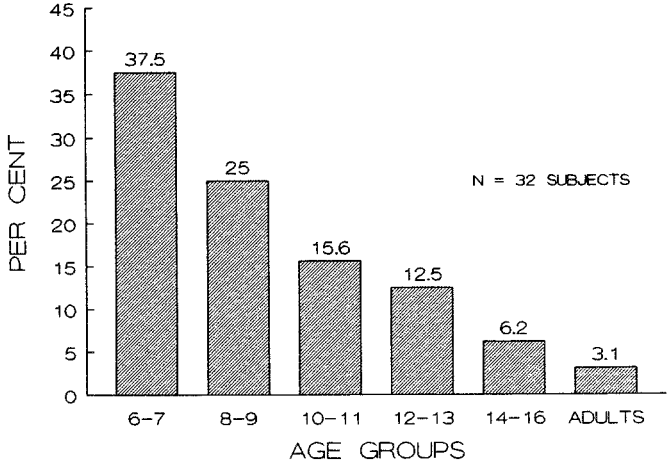

FIG. 3. Distribution of highly sensitive group (scores 31-37) by age.

TABLE 1. CHILDREN'S SENSITIVITY TO ODOR OF TRIMETHYLAMINE[a]

		All subjects				Low-sensitives only			
Sex	Age	M	SEM	SD	*N*	M	SEM	SD	*N*
Female	6-7	28.1		5.2	18				
Male		28.2		5.4	17 NS*				
Both		28.2	0.91	5.3	35	24.6	0.41	2.0	23
Female	8-9	24.5		3.7	26				
Male		25.1		4.3	36 NS*				
Both		24.8	0.52	4.1	62	23.5	0.27	1.9	54
Female	10-11	25.7		4.5	19				
Male		25.3		3.0	18 NS*				
Both		25.5	0.64	3.9	37	24.2	0.40	2.2	32
Female	12-13	24.9		3.2	32				
Male		25.3		4.0	17 NS*				
Both		25.1	0.50	3.5	49	24.2	0.34	2.3	45
Female	14-16	24.9		1.9	39				
Male		25.0		2.8	33 NS*				
Both		24.9	0.28	2.3	72	24.7	0.22	1.9	70
Female	Adults	24.1		3.0	18				
Male		24.9		2.7	10 NS*				
Both		24.4	0.56	2.9	28	24.1	0.50	2.6	27
Female	6-16	25.4		3.7	134				
Male		25.6		4.0	121 NS*				
Both		25.5	0.24	3.9	255				

[a] Arithmetic means (M), standard errors of the mean (SEM) and standard deviations (SD) for different age groups in the sample. *: *t* tests showed no significant sex differences.

Of the 288 pupils in the experimental group, 33 subjects failed to reach a TMA score. These were given the score 18, representing the strongest TMA concentration in this study and were included in the analyses of possible effects of food intake, spice, smoking cigarettes, and influenza on the sensitivity to the odor of TMA. None of the included variables were found to have a significant effect on TMA sensitivity.

Questions about smoking were only given to subjects who filled out a questionnaire on fish food preferences and rejections. Children given these questions at an interview normally were less than 13 years of age and were considered too young for smoking to be a relevant factor. One hundred twenty-six subjects (28 adults and 98 pupils ages 12–16) were asked about smoking habits, including smoking the last 60 min before testing. No significant interactions between smoking habits and TMA sensitivity were found. Adult smokers did not differ from nonsmokers concerning TMA sensitivity.

Food spice habits, as reported by the subjects, showed no significant variations with TMA sensitivity, nor did the use of lozenges, chewing gum, or food and fluid intake the last 60 min before testing. Quite surprisingly, the same lack of significant data was obtained when the sensitivity of children reporting a stuffy nose was compared with the sensitivity of subjects reporting not having stuffy noses.

Data on fish food preferences and aversions, as well as data on the frequency of fish dinners and amount of fish consumed, showed no connections with the TMA sensitivity data and will be published elsewhere.

DISCUSSION

The present study was carried out as a field experiment to ensure the accessibility of an adequate number of child subjects. The procedure was neither time consuming, frightening, nor too dependent on equipment. The chosen sensitivity criteria made similar experimental conditions available to both children and adults. The use of halving steps in the dilution series was chosen because this logarithmic scale gives the smell sensitivity distribution a nearly Gauss-shaped curve in a normal population without anosmic persons (Amoore et al., 1968).

We do not claim to have found optimal sensory thresholds, but a measure of the lowest concentration detectable under the given conditions. The lack of laboratory conditions and a significant difference in the dilution series procedure makes a direct comparison with the data reported by Amoore and Forrester (1976) difficult. A rather large part of the discrepancy in reported sensitivity measures between these studies is supposed to be due to the use of a saturated solution of TMA hydrochloride as the starting point for the dilution series in

the present study, while Amoore and Forrester (1976) refined their TMA by first extracting the free base from the hydrochloride with mineral oil.

Tereshchenko and Chemeris (1975) have reported the solubility of the hydrochloride to be 214 g/100 g water (corresponding to 1.3 million ppm TMA), while the Merck Index (1983) indicates a lower solubility than TMA without quoting values. Personal communication with Fluka Chemie AG, Switzerland, has supported the latter reference, indicating a solubility of 1 g TMA hydrochloride in 10 ml water (62,000 ppm TMA). Judged on a qualitative basis, TMA hydrochloride appears as less odor-intense than the free base (Merck Index, 1983).

The size of the air–liquid interface area will most likely also be of some importance to the degree of gas exchange and the level of gas concentration in the testing vessels. Amoore and Forrester (1976) presented their odorants in triangular shaped flasks which provided an estimated 15-fold larger gas evaporation surface compared to our test tubes. The distance from liquid level to the rim was roughly the same (9–11 cm), although the evaporated TMA contained in an Erlenmeyer flask will distribute in an air volume about four times that of a tube. The differences in testing vessel geometry could therefore account for differences in detection threshold ppm, given that the TMA concentration in the gas phase might have appeared more intense in the previous study. Nonconstant factors such as temperature fluctuations and shaking of the vessel will also influence the gas concentration, since TMA is a rather volatile substance in solution.

The present results show a strong correlation between age and TMA sensitivity, the youngest being most sensitive and adults least sensitive to the TMA smell, supporting a general notion within psychology and medicine that olfaction is weakened with age. Venstrom and Amoore (1968) reported a 50% decrease in sensitivity to 18 odorants over a 22-year adult age span in a cross-sectional study. Another cross-sectional study including 1.5 million readers of the *National Geographic* magazine also showed the smell sensitivity to six odorants to decrease with age (Gilbert and Wysocki, 1987).

Our data suggest that the TMA sensitivity only shows minor changes through the age group 8–16 years. This group was not significantly different from adults, although a decreasing tendency in sensitivity could be seen. These data indicate a similar tendency as those presented in *National Geographic* (Gilbert and Wysocki, 1987), where odor sensitivity only showed a slight fall for the ages 10–30 years.

When subjects with sensitivity scores 31–37 were removed from the analyses, age differences disappeared, showing that an unequal distribution of "highly sensitive" subjects in the different age groups accounts for most of the TMA sensitivity differences in this study. The mechanism for this unequal distribution, with a substantial proportion of highly sensitive subjects in the young-

est age groups, deserves investigation. One hypothetical explanation might be that a general wear-and-tear of the rhinal mucosa occurs with increasing age, which again might decrease the number of odor-sensitive persons and therefore lowers the average group sensitivity. Little is known about what is actually causing olfactory sensitivity changes in children and adolescents. Long-term studies should be carried out controlling for neurophysiological changes in the olfactory sense as well as for environmental and personal variables.

Our 8- to 9-year-old age group obtained a somewhat lower, although not significantly different, TMA sensitivity measure than did older groups of children. After removing high scorers from this group, the results became significantly different from the 6- to 7- and 14- to 16-year-old age groups. The 8- to 9-year-old age group consisted of pupils from both different schools and different classes, as did the other age groups. No significant differences between the classes in the 8- to 9-year-old year group were found.

Smoking was not found to interfere with the sensitivity to TMA. The lack of effects from smoking in general and smoking before sensitivity testing in particular have also been reported by Pangborn et al. (1967), Venstrom and Amoore (1968), and Vierling and Rock (1967). Whether or not smoking has an effect depends on the amount and duration of the smoking habit (Gilbert and Wysocki, 1987; Murphy, 1987). It is unlikely that smoking has had any significant effects on the results of our age groups.

Of the child subjects, 11.5% failed to have their sensitivity measure established. Some of these may be anosmic to the TMA odor, as reported by Amoore and Forrester (1976). Others may have lost their attention or interest in the test situation.

Our data clearly showed that the sexes do not differ in TMA sensitivity in any of the age groups studied. Neither Leonardos et al. (1969) nor Amoore and Forrester (1976) have reported results showing sex differences in TMA sensitivity. Our data do not support the possibility that TMA has a sexual signal function for humans as has been indicated for the red fox (Albone and Fox, 1971). The possibility remains that TMA has other signal functions perhaps tied to food poisoning. If man has a special sensitivity for TMA, as suggested by Amoore and Forrester (1976), it also may have a function in relation to avoiding contaminated fish food.

Norwegian fish food control authorities have established criteria for TMA-nitrogen concentrations in fish, allowing no more than 3 mg/100 g of cooled raw muscle and 5 mg/100 g of frozen muscle (Fiskeridirektoratet, 1986, pp. 41–42). Nitrogen constitutes 27.3% of the TMA molecular weight (The Merck Index, 1983), this being equivalent to 12.7 and 21.1 mg TMA (including minor amounts of mono- and dimethylamine) in 100 g of fish, respectively. This maximum concentration corresponds to 127 ppm (w/w) TMA in fish muscle. A considerable amount of the TMA will escape during meal preparation, either to

the air or to the water in the pan. If 1 kg of saltwater fish is cooked, the TMA concentration in the air most likely will exceed the average TMA odor detection threshold of both children and adults.

No relations between TMA sensitivity scores and frequency of fish dinners or fish food preferences and aversions were found in this study. Further studies should be carried out concerning children's subjective ratings of the TMA odor, this variable most likely being more important for fish food acceptability than the sensitivity per se.

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SURFACE LIPIDS OF THE SOCIAL WASP *Polistes annularis* (L.) AND ITS NEST AND NEST PEDICEL

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Abstract—Cuticular lipids were recovered from *Polistes annularis* workers and characterized by combined gas-chromatography–mass spectrometry. These lipids were found to be straight-chain and methyl-branched hydrocarbons with the major components being 13,17-dimethylhentriacontane (18%), 3-methyl-nonacosane (13%), and 3-methylheptacosane (11%). Cuticular lipids with a very similar composition were found on the surface of the larvae, eggs, and adult males of *Polistes annularis* as well as on the surface of the nest and nest pedicel of the wasp. Hexadecanoic acid and octadecenoic acid were also found on the surface of the nest pedicel and these acids and/or the hydrocarbons may play a defensive role for the wasps.

Key Words—*Polistes annularis*, Hymenoptera, Vespidae, defensive mechanisms, necrophoric behavior, nest pedicel, social wasps, wasps.

INTRODUCTION

Cuticular lipids protect insects from dehydration and usually have a composition that is species-specific (Howard and Blomquist, 1982; Blomquist and Dillwith, 1985; Hadley, 1985; Lockey, 1988). Cuticular hydrocarbons may also serve as recognition factors, especially among social insects (Howard et al., 1982; Post and Jeanne, 1984; Blum, 1987; Bonavita-Cougourdan et al., 1987; Vander Meer et al., 1989). Other than ants and bees, the surface lipids of relatively few members of the order Hymenoptera have been examined. Cuticular hydrocarbons have been characterized for the digger wasp, *Bembix pruinosa* (Hadley et al., 1981); the social wasp, *Parachartergus aztecus* (Espelie and

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Hermann, 1988); and the parasitoid, *Ascogaster quadridentata* and the hyperparasite, *Perilampus fulvicornis* (Espelie and Brown, 1990). In the present paper, we report on the surface chemistry of workers, males, eggs, and larvae of the social wasp, *Polistes annularis*. Additionally, we characterize lipids recovered from the surface of the nest and the surface of the nest pedicel of this wasp. The components recovered from the nest paper may aid workers in kin recognition, and the components found on the pedicel may act as deterrents to predators.

METHODS AND MATERIALS

Insect Extractions. Wasps and nests were collected in the spring, summer, and fall of 1988 and the spring and summer of 1989 in various locations around Athens, Georgia. Wasps were frozen and then extracted individually in redistilled hexane for 60 sec at room temperature. Larvae, pupae, and eggs were removed from the nests and then frozen and extracted in the same manner with hexane. Surface lipids were recovered from the nests by directing a stream of hexane at the nest surface. Nest pedicels were carefully severed from the nests in the field and immediately immersed in hexane for 60 sec. These hexane extracts were kept on ice and returned to the laboratory. Brief hexane extractions were utilized in each case in an effort to remove primarily surface lipids and to minimize the extraction of internal components (Blomquist et al., 1987). The abdominal sternal glands were excised from *P. annularis* workers, which were collected in the late spring of 1989. The glands were lyophilized and then extracted in hexane for 60 sec at room temperature.

Analysis. The various hexane extracts were concentrated under a stream of nitrogen and analyzed on a Hewlett Packard 5890A gas chromatograph with either a 12-m or a 30-m cross-linked methyl silicone capillary column (0.2 mm internal diameter; 0.33 μm film thickness) with helium as the carrier gas. The column was held at 55°C for 3 min after injection (splitless), and then the temperature was increased to 305°C at a rate of 25°C/min. The column was connected to a mass spectrometer (Hewlett Packard 5970), and mass spectra were recorded at 70 eV at 1.3-sec intervals. Components were characterized by analysis of their mass spectra and determination of their equivalent chain lengths (Nelson, 1978; Blomquist et al., 1987); quantitation was based upon peak areas of total ion chromatograms of standard hydrocarbons and fatty acids (Sigma Chemical Co.) (Espelie and Bernays, 1989).

Mass spectra and GC retention times of hexadecanoic and octadecenoic acids recovered from the nest pedicels and sternal glands were identical to those of authentic standards. Fatty acids recovered from the nest pedicels were converted to methyl esters by refluxing for 2 hr in 14% BF_3/methanol and char-

acterized by GC-MS and found to be identical to authentic standards (Espelie et al., 1983). Double-bond location was accomplished by GC-MS analysis of epoxides generated by reaction with *m*-chloroperoxybenzoic acid (Baker et al., 1984).

Microscopy. Pedicel and nest paper samples were mounted on aluminum stubs adhered by silver paint. A Hummer X Sputter Coater (Anatech Ltd.) was utilized to apply a 50-nm coat of gold–palladium on the samples. A Phillips 505 Scanning Electron Microscope, with an accelerating voltage of 20 kV, was used for observing and photographing samples.

RESULTS

The surface lipids of *P. annularis* workers were found to be a complex mixture of *n*-alkanes, monomethylalkanes, and dimethylalkanes. The major components were 13,17-dimethylhentriacontane (18%), 3-methylnonacosane (13%), 3-methylheptacosane (11%), and *n*-heptacosane (8%) (Table 1). A dominant feature of the mixture of cuticular hydrocarbons was the large number of 3-methylalkanes that are readily characterized by their mass spectra. In each spectrum, there was a major fragment at M−29 due to cleavage between C_2 and C_3 and a smaller fragment at M−57 resulting from cleavage between C_3 and C_4 (Figure 1). The cuticular lipids of the workers contained six 3-methylalkanes with a carbon backbone ranging in size from C_{23} to C_{31} comprising 27% of the total hydrocarbon complement.

The cuticular hydrocarbons of adult male *P. annularis* were the same as those found on the surface of the workers. There were, however, several components present in amounts that differed from those recovered from the workers (Table 1). The major component in the male cuticular lipids was 3-methylnonacosane (21%), while 13,17-dimethylhentriacontane, the largest component in the worker cuticular lipids, comprised only 3.5% of the male hydrocarbons. The cuticular lipids recovered from the eggs and larvae of *P. annularis* also contained the same components found on the workers and with very similar distribution patterns (Table 1). 3-Methylnonacosane was the largest component (23%) in the cuticular lipid fraction of the eggs, while 13- and 15-methylnonacosane was the largest peak (15%) in the hydrocarbons recovered from the surface of the larvae.

The hexane extract of *P. annularis* nest paper contained primarily hydrocarbons that were the same as those recovered from the cuticle of the workers, adult males, larvae, and eggs (Table 1). The major components had a backbone of either C_{27} or C_{29} (Figure 2A). Similarly, the hexane extract of the pedicel of *P. annularis* nests contained the same hydrocarbons found in the wasps' cuticle (Table 1). There was a higher proportion of longer chain-length components

TABLE 1. CUTICULAR LIPID COMPOSITION OF *Polistes annularis* WORKERS, MALES, EGGS, LARVAE, NEST PAPER, AND NEST PEDICEL[a]

TIC peak	Component	Composition (%)					
		Workers	Males	Eggs	Larvae	Nest paper	Pedicel
1	Hexadecanoic acid						9.9
2	Octadecenoic acid						17.7
3	*n*-Tricosane	0.5				0.6	0.4
4	*n*-Pentacosane	0.6	0.5		3.2	1.4	0.6
5	3-Methylpentacosane	0.8				0.5	
6	*n*-Hexacosane	0.4	0.1			0.8	0.3
7	*n*-Heptacosane	8.1	4.7	8.7	14.7	12.1	4.5
8	11- and 13-Methylheptacosane	1.5	7.2	1.9	4.4	10.1	7.2
9	5-Methylheptacosane		1.2			1.7	0.9
10	3-Methylheptacosane	10.7	10.3	12.5	11.9	10.4	6.9
11	*n*-Octacosane	1.4	0.8	5.8	3.1	2.0	0.5
12	X-Methyloctacosane		1.8			2.2	1.1
13	2-Methyloctacosane	0.7	1.7	2.6	0.5	2.1	1.6
14	3-Methyloctacosane	0.5	1.1		2.8	1.1	0.2
15	*n*-Nonacosane	3.1	3.2	8.9	6.9	5.7	2.7
16	13- and 15-Methylnonacosane	4.8	16.8	7.3	15.2	13.5	15.2
17	13,17-Dimethylnonacosane	6.1	3.7		6.5	4.8	6.1
18	3-Methylnonacosane	13.2	21.4	22.9	12.6	12.2	6.0
19	2-Methyltriacontane	1.9	1.1	2.0	1.1	0.9	1.0
20	13- and 15-Methylhentriacontane	4.7	8.9	6.0	4.9	3.8	6.8
21	13,17-Dimethylhentriacontane	17.6	3.5	15.4	8.6	2.4	6.7
22	3-Methylhentriacontane	1.5	0.5	1.1		0.5	0.6
23	11-, 13-, 15- and 17-Methyltritriacontane	1.2					
24	15,19-Dimethyltritriacontane	6.5	6.1			1.5	

[a]Total ion chromatogram (TIC) peak numbers correspond to those in Figure 2. Components are listed only if their presence was confirmed by mass spectral analysis.

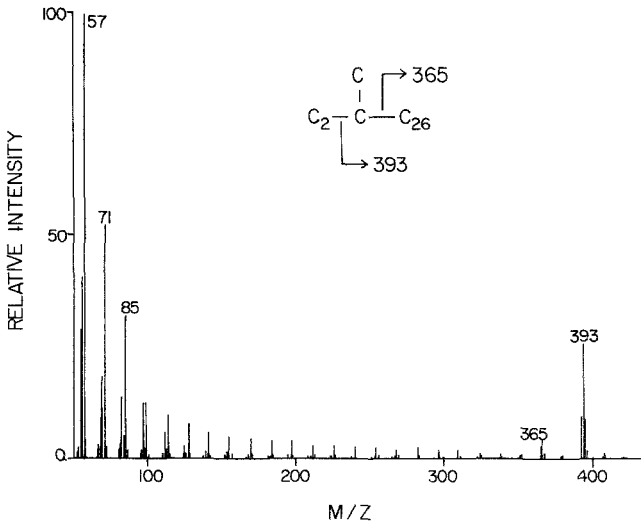

FIG. 1. Mass spectrum of 3-methylnonacosane, a major component of the cuticular lipids of *Polistes annularis* workers.

(C_{31}) and a lower proportion of shorter chain-length components (C_{27}) among the hydrocarbons found on the pedicel versus those recovered from the nest paper. However, the most dramatic difference between the lipids recovered from the two portions of the nest was the presence of two components extracted from the pedicel: peaks 1 and 2 in Figure 2B. These components were shown by their mass spectra and by GC-MS analysis of authentic samples to be hexadecanoic acid and octadecenoic acid, respectively. The fatty acids were converted to methyl esters by treatment with BF_3–methanol and shown by GC-MS analysis to be methyl hexadecanoate and methyl octadecenoate.

The amount of total lipid recovered from the pedicel was determined by integration of the total ion chromatograms and was found to be much greater than that recovered from the nest paper or that extracted from the wasps, larvae, or eggs. The extractable lipids comprised more than 4% (on a weight basis) of the pedicel (Table 2). The larvae, conversely, had the lowest proportion of extractable lipids: less than 0.002% on a fresh weight basis.

Pedicels and nest paper were examined by scanning electron microscopy before and after hexane extraction. Prior to extraction, the pedicel had a relatively smooth amorphous apearance (Figure 3A). After hexane extraction, however, a series of parallel lines could be seen that were perpendicular to the axis of the pedicel (Figure 3B). The surface of the nest paper had an appearance that was very different from that of the pedicel; the surface was dominated by a large number of fibers of varying size (Figure 3C). Extraction of the nest paper with hexane had no effect upon the ultrastructural appearance.

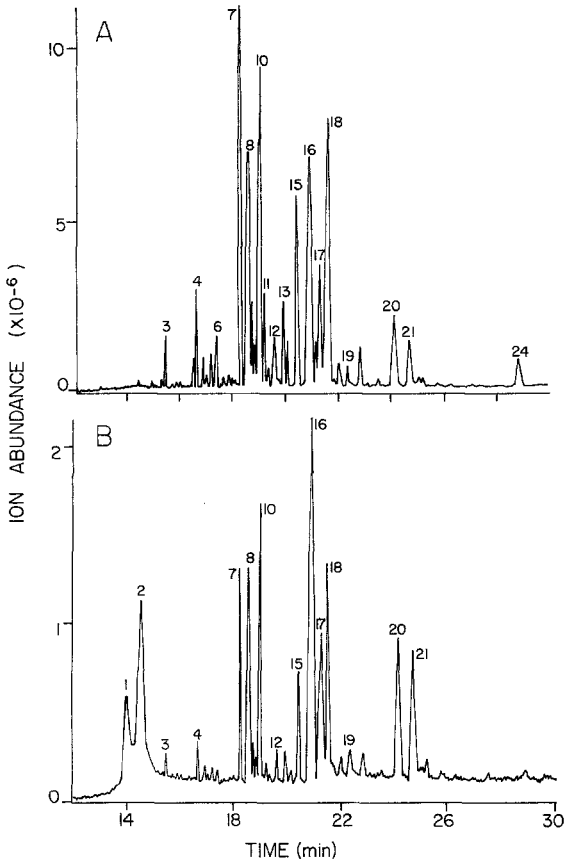

FIG. 2. Total ion chromatograms of the hexane extracts of: (A) the nest paper of a nest of *Polistes annularis* and (B) the pedicel of the same nest. Numbered peaks were identified by their mass spectra and are listed in Table 1.

TABLE 2. TOTAL SURFACE LIPIDS RECOVERED BY HEXANE EXTRACTION OF *Polistes annularis* WORKERS, MALES, EGGS, LARVAE, NEST PAPER, AND NEST PEDICEL

Polistes annularis	Total lipid (μg/mg fresh wt)
Workers	4.70
Males	3.84
Eggs	4.50
Larvae	0.02
Nest paper	2.10
Nest pedicel	44.00

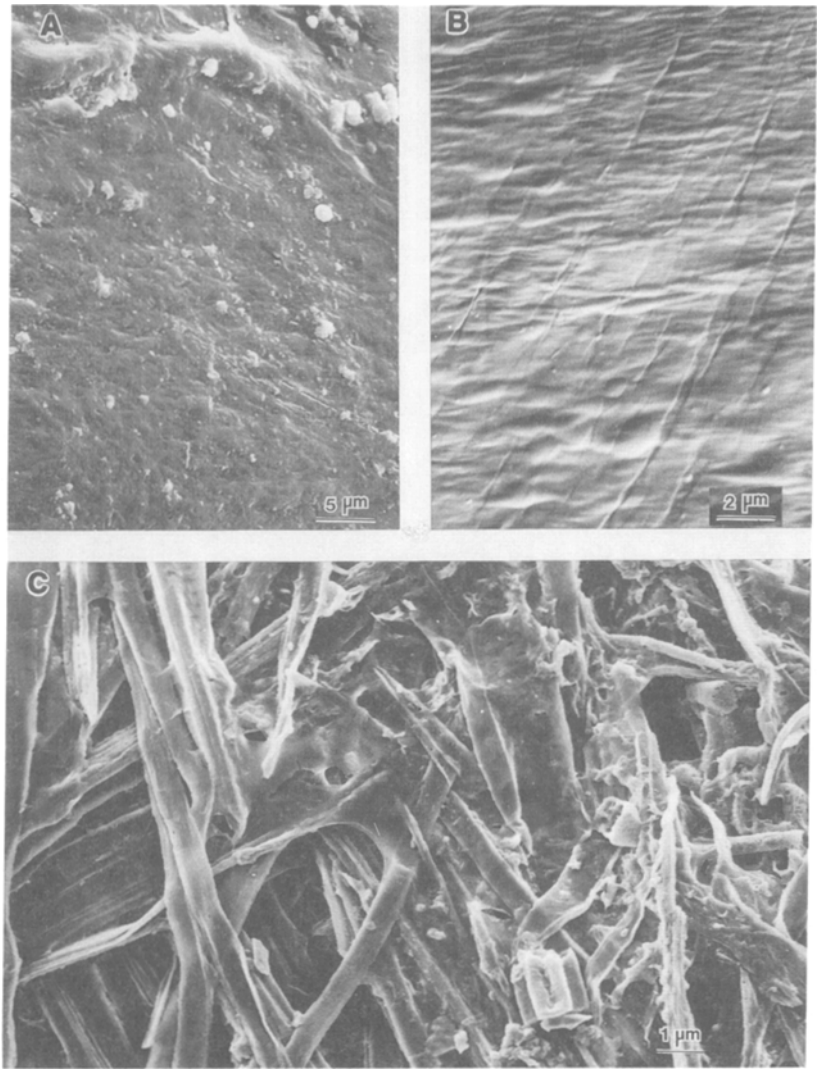

FIG. 3. Scanning electron micrographs of: (A) the pedicel from a nest of *Polistes annularis* before hexane extraction; (B) the pedicel from a nest of *Polistes annularis* after hexane extraction; (C) the outer surface of the paper of the main body of the nest.

The van der Vecht's gland, located beneath the terminal gastral sternite segment, was excised from *P. annularis* workers collected in the late spring. These glands were dried and then extracted with hexane. The hexane extracts were analyzed directly by combined GC-MS. Several components, which were

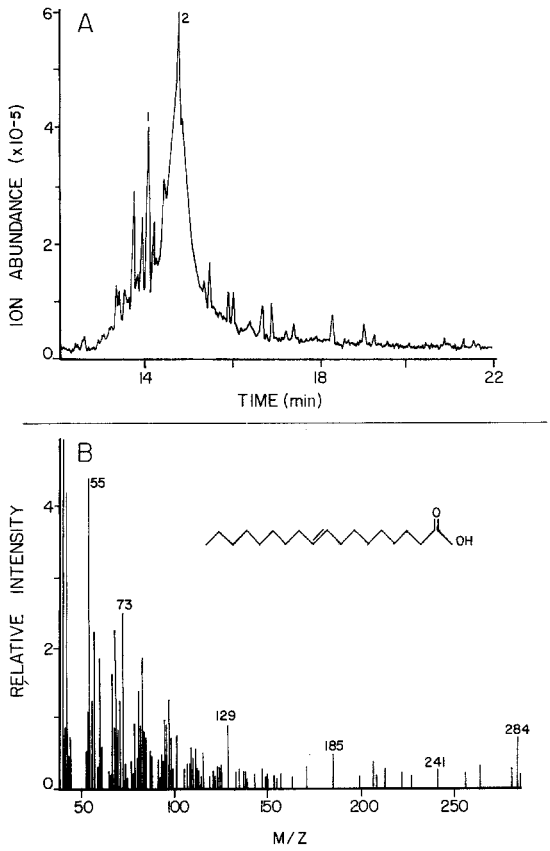

FIG. 4. (A) Total ion chromatogram of the hexane extract of excised sternal glands from seven workers of *Polistes annularis*. Peaks 1 and 2 were shown by their mass spectra to be hexadecanoic acid and octadecenoic acid, respectively. (B) Mass spectrum of the major component of the hexane extract of the sternal glands, octadecenoic acid (peak 2 in panel A).

poorly resolved, eluted as shown by the total ion chromatogram (Figure 4A). Peaks 1 and 2 were identified by their mass spectra as hexadecanoic acid and octadecenoic acid, respectively. Selective ion scanning for major ions observed in the mass spectrum of authentic octadecenoic acid (Figure 4B) indicated that this fatty acid was the major component in the broad peak 2 of Figure 4A. Selective ion scanning was also employed in an effort to detect the presence of the methyl esters of hexadecanoic and octadecenoic acids. Neither of these esters were detected in the hexane extracts of the *P. annularis* sternal glands.

DISCUSSION

The similarity in cuticular hydrocarbon composition of larvae and adult female and male *P. annularis* is a feature that has been observed for the cuticular lipids of several other insects when the surface lipids of both larvae and male and female adults have been examined (Blomquist and Dillwith, 1985; Lockey, 1988). However, the similarity between the cuticular lipids of eggs and those of larvae and adults of the same species has previously been reported only for *Manduca sexta* (Nelson et al., 1972). Although 3-methyl alkanes are common components in insect cuticular lipids (Blomquist et al., 1987; Lockey, 1988), the amount found in the cuticular lipids of *P. annularis* workers (27%) is relatively high.

Jeanne (1970) reported that the social wasp, *Mischocyttarus drewseni*, smears the pedicel of its nest with a secretion from the sternal gland, which serves as a deterrent to predators. The presence of this gland and the utilization of this defensive behavior have subsequently been reported for several genera of social wasps (Hermann and Dirks, 1974; Landolt and Akre, 1979; Delfino et al., 1979; Turillazzi and Ugolini, 1979; Post and Jeanne, 1980, 1981; Kojima, 1982, 1983; Jeanne et al., 1983). Methyl hexadecanoate has been recovered from an extract of the sternal gland of *P. fuscatus* workers and shown to act as a deterrent to several species of ants (Post et al., 1984; Henderson and Jeanne, 1989). The free fatty acids (C_{16} and $C_{18:1}$) found on the pedicel of *P. annularis* nests (Figure 2B) and extracted from the sternal glands of workers (Figure 4A) might also act as deterrents to ant predators. These fatty acids have been shown to elicit a necrophoric response in ants (Wilson et al., 1958; Blum, 1970; Haskins and Haskins, 1974; Howard and Tschinkel, 1976; Gordon, 1983) and, therefore, ants might be especially reluctant to cross over a pedicel containing these compounds. Ants are the major predators of social wasps (Jeanne, 1975) and in the New World this predation becomes greater closer to the equator (Jeanne, 1979). It is likely that social wasps synthesize a variety of repellents to deter ants, and these repellents may need to be more potent in the tropics in order to overcome increased predation pressure.

The amorphous appearance of the *P. annularis* nest pedicel as seen in the scanning electron micrograph (Figure 3A) is characteristic of a surface covered with a lipid layer composed primarily of hydrocarbons (Baker, 1982; Jeffree, 1986) and is very similar to that shown for the nest pedicel of the social wasp, *Mischocyttarus mexicanus cubicola* (Hermann and Chao, 1984). The large amount of hydrocarbons found on the surface of the pedicel (Table 2) might serve as a means of defense by notifying predators of the wasps' presence. The parallel strips seen after hexane extraction of the pedicel may be the layers of material laid down by the wasp during pedicel construction (Figure 3B). The

surface of the pedicel is clearly different from the surface of the main part of the nest (Figure 3C). The pedicel of social wasp nests is composed, to a large extent, of an oral secretion (Richards, 1971; Jeanne, 1975; Edwards, 1980). Oral secretions also are used to bind together the paper of the comb of the nest, and the recent report that an oral secretion of the wasp, *Pseudochartergus chartergoides*, contains chitin (Schremmer et al., 1985) indicates that it would be interesting to examine the chemical composition of the pedicel.

The hydrocarbons on the nest paper may serve a recognition role for the wasps. Hydrocarbons found on the nest paper of the wasp *Parachartergus aztecus* seem to play a role in allowing that wasp to nest in *Acacia* trees with aggressive *Pseudomyrmex* ants (Espelie and Hermann, 1988). The nest of several species of *Polistes* wasps has been shown to be important in establishing kin recognition in newly emerged workers (Shellman and Gamboa, 1982; Pfennig et al., 1983; Gamboa et al., 1986), and the hydrocarbons applied by the *P. annularis* wasps to the nest paper during nest construction may play a crucial role in this recognition process.

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IMPORTANCE OF QUINOLIZIDINE ALKALOIDS IN THE RELATIONSHIP BETWEEN LARVAE OF *Uresiphita reversalis* (LEPIDOPTERA: PYRALIDAE) AND A HOST PLANT, *Genista monspessulana*

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Abstract—Larvae of *Uresiphita reversalis* feed almost exclusively on legumes in the tribe Genisteae, which characteristically contain a variety of quinolizidine alkaloids. The larvae are aposematic, and on *Genista monspessulana*, a major host in California, they feed on the youngest leaves, at the periphery of the plant. These leaves, which were preferred over older foliage in choice tests, contained four to five times the level of alkaloid found in older leaves. The major alkaloids detected in these plants were dehydroaphylline and *N*-methylcytisine, together accounting for 74% of the total. Preliminary analyses showed the alkaloid profile of exuviae from larvae feeding on these plants was very similar to that of the plants. Two alkaloids, sparteine and cytisine, which are known components of some hosts of *U. reversalis*, were phagostimulants for fifth-instar larvae when added to sucrose-impregnated glass-fiber disks. In addition, when sparteine was added to foliage of *G. monspessulana*, effectively doubling the percent dry weight of alkaloid, the growth rate of late-instar larvae was positively affected. Cytisine added to plants had no discernible effect on growth of larvae. Alkaloid levels in larvae and in their frass were proportional to levels in the plants on which they fed. Although the majority of alkaloid was excreted, that which was sequestered by the insect was found entirely in the integument, possibly confering some protection from predators.

Key Words—*Uresiphita reversalis*, Lepidoptera, Pyralidae, *Genista*, French broom, quinolizidine alkaloids, sequestration, aposematism.

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INTRODUCTION

Larvae of the pyralid moth, *Uresiphita reversalis* (Guenée), have been recorded from plants in the tribe Genisteae (Papilionaceae), primarily species of *Lupinus*, *Genista*, *Laburnum*, *Cytisus*, and *Sarothamnus*. In California, the insect is most common on the exotic weed, French broom, *Genista monspessulana* (= *Cytisus monspessulanus*), although it is also found on various species of *Lupinus* and other legumes (California Department of Food and Agriculture, unpublished.)

The eggs of *U. reversalis* are laid in groups of up to 70 on the host plant. The newly hatched larvae are greenish and remain densely aggregated until the later instars. By the third instar, larvae are primarily black, while later stages are black with yellow and white marks. Larvae are almost invariably found peripherally on terminal branchlets, actively feeding throughout the day. The coloration and conspicuousness of the later-instar larvae indicate that they are aposematic (Bernays and Montllor, 1989), and there is good evidence that they are very distasteful to a variety of predators (Bernays, 1988; Bernays and Cornelius, 1989; Montllor, Bernays, and Cornelius, in preparation). In our mixed-age laboratory and greenhouse colonies only pupae were subject to predation by Argentine ants (*Iridomyrmex humilis*). Taken together, these observations suggest that the larvae have some form of chemical protection.

Since insects in the field feed on the periphery of the plant, their food consists largely of young leaves. The primary reason for this is unknown and may relate to advertisement, thermoregulation, or food quality, including nutrients and plant secondary chemistry. The species of Genisteae that are host plants for *U. reversalis* are characterized by the presence of quinolizidine alkaloids (sometimes called lupine alkaloids) (Kinghorn and Balandrin, 1984). Some of these compounds are mammalian toxins and have also been shown to have noxious effects on other plants, various herbivores, fungi, and bacteria (Wink, 1985). On the other hand, the aphids *Acyrthosiphon spartii* and *Macrosiphon albifrons*, which are specific on *Lupinus*, are stimulated to feed by the appropriate quinolizidine alkaloids (Smith, 1966; Wink and Römer, 1986). In addition, *M. albifrons* appears to be protected from beetle predators by virtue of the quinolizidine alkaloids it sequesters from its lupine hosts (Wink and Römer, 1986).

A wide variety of lepidopterans that feed on alkaloidal plants are aposematic and sequester the toxic alkaloids, thereby gaining some protection from their predators (Duffey, 1980; Rothschild, 1972). Examples of lepidopterans protected specifically by quinolizidine alkaloids are not known, however, and this study is an examination of the possible importance of these alkaloids for *Uresiphita reversalis*.

METHODS and MATERIALS

Cultures. A culture of *Uresiphita reversalis* was maintained in an insectary with a light–dark regime of 16:8 hr, and a mean temperature of 25°C. Sleeve cages were approximately 0.5 m^3 with glass tops and gauze sides. Larvae were fed on cut branches of *Genista monspessulana* standing in water, and food was changed every one to three days. Pupation occurred in the corners of the same cages, and, after emergence, adults were provided with sugar water in a flask with a cotton wick. Females laid eggs on fresh plant material and on the sides of the cages. Insects were occasionally collected from the field and added to the culture.

Potted plants were grown for some experiments in a greenhouse. These were grown from seed collected from a single plant at a field study site in Berkeley. During hotter periods, a proportion of the pots were removed to a lathe house where conditions were similar to those of the natural environment. Growth experiments with insects were carried out in both the lathe house and greenhouse.

Assay for Total Alkaloids. Alkaloid extracts of fresh and oven-dried broom (see below) were spotted on silica gel plates and chromatographed using a solvent system of cyclohexane–diethylamine (7:3). Fresh and dried material gave identical spots when visualized with Dragendorff reagent. Consequently, only dried material was used in assays for total alkaloids.

Alkaloids were extracted by standard methods (Wink et al., 1982; Dolinger et al., 1973), with some modifications. Plant and insect material was oven-dried at 70°C, ground in a Wiley mill to 40 mesh, and extracted in methanol. The dried extract was taken up in 1 N HCl, and partitioned with ether. The aqueous portion was then basified with ammonia to pH 10, extracted with chloroform, and the chloroform evaporated. The dried extract was taken up in 0.5 N HCl, and assayed by adding a 0.1-ml aliquot to 4 ml of an aqueous solution of KI/I_2 (Wink, personal communication). The absorbance of this mixture was read at 800 nm after 3 min. Sparteine was used as a standard.

Chemical Analysis of Alkaloid Profile. Chemical analyses of selected samples of *G. monspessulana* were carried out by GLC and GC-MS, and identifications of alkaloids made by EI-MS and CI-MS, according to the methods of Wink and Witte (1984).

Alkaloids in Plants and Insects. In order to get a measure of the variability of total quinolizidine alkaloid levels in *G. monspessulana*, plants were sampled in the field. Temporal variation was examined by taking repeated samples from the same seven plants over 18 months. Between-plant variation was examined in three to four plants at each of six sites, sampled within a day of each other.

In both cases, leaves from the terminal 15–20 cm of three to five branches per plant were used. Variation within plants was examined by separating foliage from two plants into younger and older foliage.

Comparisons of alkaloid levels in broom plants and *U. reversalis* were made from field and laboratory material in two separate experiments. In the first, approximately 20 fourth-and fifth-instar larvae were collected from natural populations on each of four plants at widely separated sites. Undamaged leaf material from these plants was collected at the same time and dried. Insects were starved 24 hr to allow them to empty the gut before they were dried and assayed for total alkaloids. In the second experiment, plant material was collected every other day from four plants in the field. Material from one of the plants was divided into younger and older branchlets, making a total of five treatments. Fifty third-instar larvae from a laboratory culture (reared on broom) were placed on cut branches from each plant and placed in a growth chamber at 18–24°C. Each time plant material was changed, a portion was also collected for subsequent alkaloid analysis. Larvae were removed as late fifth instars, frozen, then dissected to remove gut contents. Plants, insects, and frass from each treatment were dried and analyzed for total alkaloids.

Alkaloid levels in exuviae and in pupae were measured on two occasions several months apart. Cast exuviae from last-instar larvae were removed from the cocoons of several hundred pupae, and a subset of the pupae was also collected. Pupae and exuviae were dried and analyzed for total alkaloids.

Chemicals. Sparteine and cytisine (Sigma Chemical Co.) were used in behavior and growth studies (see below). These are known components of at least some of the hosts of *U. reversalis*. Sparteine is a major component of *Lupinus arboreus* and *Sarothamnus scoparius* (Wink and Römer, 1986), both hosts of this insect. This alkaloid has also been tentatively reported in *G. monspessulana* (Gill and Steinegger, cited in Balandrin et al., 1982), although we and others (Balandrin et al., 1982) have not detected it. Cytisine has been found by us and others in *G. monspessulana* as well as in *Laburnum* (Balandrin et al., 1982).

Behavior. In the field, larvae feed primarily on the youngest leaves of *G. monspessulana*. Two separate experiments were designed to determine whether this is due to a larval preference for leaves of a certain age and/or to phototaxis.

In the first experiment, insects were allowed to select among two age classes of *G. monspessulana*: young leaves (trifoliates) with leaflets each approximately 5 mm long, and single leaflets of fully expanded mature leaves, where each leaflet was 10–15 mm long. Three young leaves and three older leaflets were placed in a circle of alternating ages in each Petri dish (9 cm diam.), which was lined with moist filter paper. One fourth-or fifth-instar caterpillar was placed in the center. There were 10 replicates in an experiment and three experiments.

Each experiment used leaves from a different individual plant. The Petri dishes were kept in an environment room at 30°C for 2–4 hr until half the leaf pieces in each dish had been fed upon. For each larva (30 total), the leaf age class that was eaten most was visually determined and recorded.

In a second experiment, we tested whether larvae are attracted to light. Ten cut branches of *G. monspessulana* (20–30 cm long) were arranged radially with, alternately, proximal or distal ends pointing towards the center. The arrangement was set out at 30°C in a dark room with a 5-W tungsten lamp at the center of the array. One fourth-instar larva was placed on each branch half way along its length, and the movements of the larvae were monitored for 10 min. The experiment was repeated three times. Numbers of individuals moving more than 5 cm either towards or away from the light were scored.

The possible role of quinolizidine alkaloids in host-plant selection behavior was examined directly using choice tests with chemicals added to sucrose-impregnated filter disks. Glass fiber filter disks (2.1 cm diam; Whatman GF/IA) were treated with 100 μl of an aqueous sucrose solution, to give 5% dry weight, or an aqueous sucrose solution also containing the test chemical, either sparteine or cytisine, to give 5% sucrose plus 0.1–2.0% dry weight alkaloid. A pair of moist control (C) and treatment (T) filters were offered in Petri dishes (9 cm diam.). Each dish had a moistened filter paper attached to the inside of the lid.

One- to three-day-old fifth-instar larvae were taken from the colony and kept without food for one day prior to the beginning of the experiments. One larva was placed in each dish, and the dishes were returned to the insectary. The percent of each filter eaten was estimated visually approx. 24 hr (range was 19–31 hr in the different experiments) after the beginning of an experiment. For each larva, the proportion of all material eaten that came from the treatment filter was calculated as $T/(T + C) \times 100$. Each chemical was tested at four concentrations (0.1, 0.5, 1.0, and 2.0% dry weight) for a total of eight tests, and between 14 and 20 larvae were used for each test.

Behavior data were analyzed with Wilcoxon's signed-rank test, or the sign test.

Growth. Low concentrations (0.02% dry weight) of lupine alkaloids were found to have a detrimental effect on the generalist lepidopteran *Spodoptera eridania* when incorporated into artificial diets (Johnson and Bentley, 1988), but specialists on Genisteae would be expected to be tolerant of these compounds. This was examined in *U. reversalis* by experiments with larvae over one or two instars. The long-term effect of moderately high alkaloid levels on growth was tested by adding alkaloids to host plants. Our assays showed that levels of quinolizidine alkaloids in *G. monspessulana* in the San Francisco Bay area varied from less than 0.1% dry weight to over 1.5%. We aimed to add

approx. 1% dry weight alkaloid to the experimental plants, more or less doubling the average alkaloid concentration in the food. Experiments were carried out with added sparteine and cytisine.

A pair of branches was chosen on each of 20 potted broom plants, approximately 50 cm high. One of the pair was dipped in 70% ethanol with the alkaloid, and the other in aqueous ethanol alone. Sparteine-treated and control branches on an additional 10 plants were collected immediately after treatment (five plants) and one week after treatment (five plants) to determine how much alkaloid was actually added, and if losses occurred over time. Branches that were sampled immediately after solvent or alkaloid treatment had a mean of 0.4% and 0.7% dry weight alkaloid, respectively. The branches sampled one week after solvent or alkaloid treatment had a mean of 0.7% and 1% alkaloid, respectively. Therefore, we could assume that added sparteine substantially increased the alkaloid level in or on leaves of the experimental plants and that this increase persisted for at least the first week after treatment, during which consumption of foliage by larvae was greatest. We also assumed that cytisine treatment gave similar results, although this was not tested separately.

Newly molted fourth-instar (cytisine experiment) or fifth-instar (sparteine experiment) larvae were paired by weight and placed individually in mesh bags on treated and control branches in a lathe house. Average temperatures in the lathe house were 18°C day and 12°C night. After pupation, final fresh weights were taken and frass was collected from each bag. Relative growth rates (RGR) (mg/mg/day) and relative egestion rates (RER) (mg/mg/day) were calculated over the fifth instar using fresh weights and exponential mean weights (Montgomery, 1983) for larval RGRs, and frass dry weights for RERs. Data were analyzed using Wilcoxon's signed-rank test.

RESULTS

Chemistry. Twenty-six alkaloids were detected in a single sample of French broom. The major alkaloid was tentatively identified as dehydroaphylline, and this accounted for approximately 46% of the total alkaloid fraction. *N*-Methylcytisine accounted for 28%, and aphylline, epiaphylline and virgiboidine each occurred at 5% or greater of the total. The details on these will be published elsewhere (Wink, in preparation).

Alkaloids in Plants and Insects. Total alkaloid levels varied within individual plants over two seasons without a discernible pattern. Up to fivefold differences were measured in the same plant sampled on different dates, although most differences were more moderate. Within a sampling date, differences among plants were on the order of two- to fivefold (Figure 1). A similar level of variation between plants was seen within and between different sites (Figure 2).

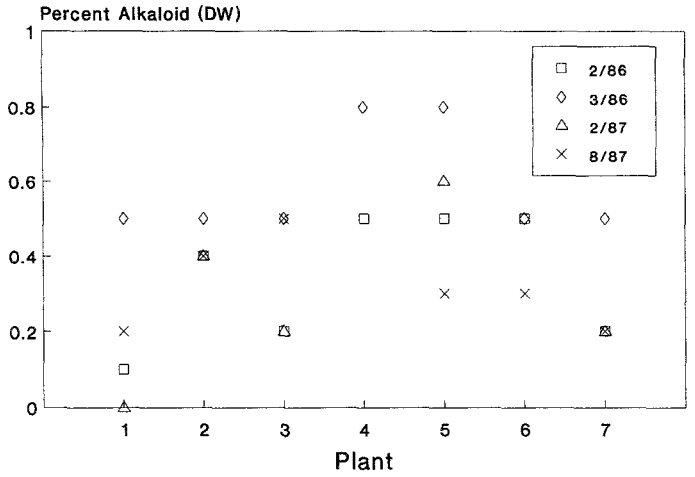

FIG. 1. Total alkaloids (% DW) in seven *G. monspessulana* plants at a single site, sampled repeatedly over 18 months.

Alkaloid levels in young foliage were found to be four to five times higher when compared to older foliage on the same branches. Comparisons were made on two plants, yielding 0.4% and 0.1% (dry weight) alkaloid in young vs. old leaves of one plant, and 1% and 0.2%, respectively, in a second plant.

A highly significant linear relationship existed between levels of alkaloids in broom plants and levels in larvae that had fed on these plants ($r = 0.96$,

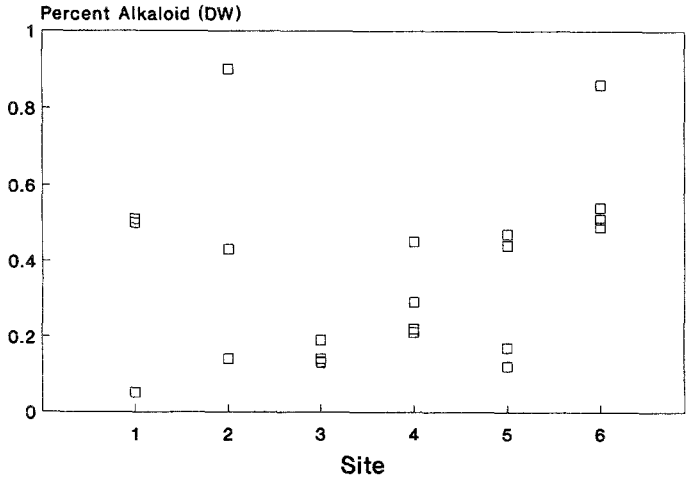

FIG. 2. Total alkaloids (% DW) in three to four *G. monspessulana* plants at each of six sites in the San Francisco area.

$P < 0.001$; Figure 3A), as well as between levels in plants and frass ($r = 0.91$, $P < 0.01$; Figure 3B). Larvae contained, on average, one fifth the level of alkaloids found in the plants they ate, while their frass contained 1.4 times the level in the plant material.

The mean level of alkaloids in late-instar larvae in these experiments was 0.11% (dry weight). Levels in two separate samples of exuviae were 2.5% and 2.6%. The dry weight of a fifth-instar larva at pupation is approximately 15 mg. The average dry weight of an exuviae is 0.7 mg. Therefore, on a dry-weight basis, it is estimated that the amount of alkaloid in a larva and in an exuviae is virtually identical, 17–18 μg. Based on this calculation, and the fact that alkaloid levels were virtually undetectable in pupae (<0.01%), it appears that consumed alkaloids that are not excreted are stored in the larval cuticle. Preliminary data indicate that the major alkaloids in the plant and insect are the same (Kubo, unpublished).

Behavior and Growth. Larvae invariably preferred younger leaves over older ones when given a choice. In each of the three experiments, most indi-

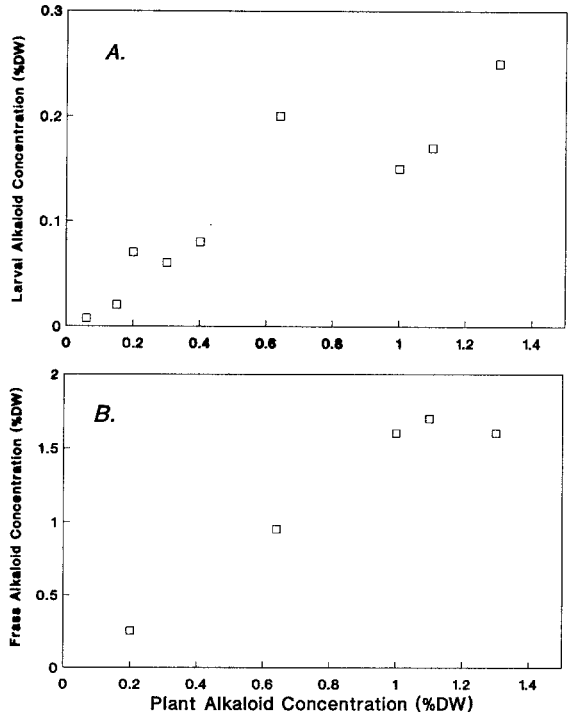

FIG. 3. Total alkaloids (% DW) in *U. reversalis* larvae (A), or frass of larvae (B) fed on *Genista* foliage with varying mean concentrations of alkaloids.

viduals clearly ate more of the younger leaves (sign test on pooled experiments, $N = 30$, $P < 0.001$). In addition, all larvae were positively phototactic. All 30 individuals moved towards the light, and 29 reached the tip of the branch nearest the light. The relative importance of these two mechanisms is unknown, but it is clear that larvae do prefer the younger leaves, and it is possible that the phototactic behavior helps larvae to find them.

Last-instar larvae of *U. reversalis* given a choice of sucrose-treated or sucrose+cytisine-treated glass fiber disks ate significantly more of the disks treated with 0.1, 1, and 2% cytisine (Table 1). They ate more of the disks treated with 0.5% cytisine, but this difference was not significant. No differential response to the increasing concentrations of alkaloid used was evident.

Larvae ate more of sparteine-treated disks than control disks, although the difference was not significant at any one of the four concentrations of sparteine used (0.1, 0.5, 1, and 2% dry weight). However, when all treatments were pooled, there was a significant preference for sparteine-treated disks compared to sucrose controls (sign test, $N = 61$, $P < 0.05$). Overall, the two alkaloids tested are demonstrably phagostimulatory, with cytisine having a greater effect than sparteine.

Pupal weights and relative growth rates (RGR) of insects fed sparteine-treated broom foliage during the fifth instar were significantly higher than those of insects fed untreated foliage on the same plants (Table 2). There were no significant differences in the developmental time (duration of the fifth instar) or in the relative egestion rate (RER) for insects on alkaloid-treated compared to control plants. There were no significant differences in any of these parameters

TABLE 1. MEAN PERCENTS OF GLASS FIBER DISKS EATEN BY *U. reversalis* IN CHOICE TESTS[a]

Dose (% dry wt)	N	Mean $T/(T + C) \times 100$	P (sign test)
Sparteine			
0.1	15	65	>0.05
0.5	14	54	>0.05
1.0	18	55	>0.05
2.0	14	71	>0.05
Cytisine			
0.1	14	91	<0.01
0.5	16	72	>0.05
1.0	19	95	<0.001
2.0	15	89	<0.01

[a] T = 5% sucrose + alkaloid; C = 5% sucrose alone.

TABLE 2. MEANS (RANGE) OF GROWTH PARAMETERS OF LARVAL *U. reversalis* FEEDING ON BROOM FOLIAGE WITH OR WITHOUT ADDED ALKALOID[a]

Treatment	*N*	Pupal wt (mg)	RGR (×100)	Days in 5th instar	RER
Sparteine	20	119.1* (100–142)	4.8* (3.3–6.2)	14.4	0.20
Control	20	113.0 (94–129)	4.5 (1.9–6.3)	14.4	0.19
Cytisine	12	117.0 (59–147)	3.1 (0.6–4.1)	23.1	0.10
Control	12	130.6 (82–160)	3.3 (1.1–4.6)	23.8	0.10

[a]RGR = relative growth rate; RER = relative egestion rate. *$P < 0.05$, Wilcoxon's sign-rank test.

measured for larvae feeding on cytisine-treated compared to control broom foliage (Table 2).

DISCUSSION

The results suggest that quinolizidine alkaloids are important in the biology of *U. reversalis*. Host plants contain alkaloids at levels generally between 0.1 and 1.5% of the dry weight of the foliage, with the higher concentrations in the younger, preferred leaves. Two typical host-plant alkaloids were found to be phagostimulatory, and one of them had a positive effect on growth rate. The ingested alkaloids that were measured in the insect after emptying of the gut were wholly accounted for by the levels found in the cuticle. At pupation virtually all alkaloids were lost with the exuviae.

The alkaloids present in *G. monspessulana* around the San Francisco Bay area were similar to those found for this species collected elsewhere (Balandrin et al., 1982). In northern California, dehydroaphylline is the major component, whereas aphylline has been shown to be dominant in other areas (Balandrin et al., 1982). Within- and between-plant variation is of a similar order to that found in other plants containing such compounds (Johnson et al., 1987; Wink and Witte, 1984) and the patterns in general, such as decrease in alkaloid levels in older leaves, are also similar to other alkaloidal plants (McKey, 1979).

The alkaloid profile in insect exuviae was similar to that of the foliage on which they fed. The amounts sequestered by the insect represented only a small proportion of the amounts ingested; most could be recovered in the frass. Assuming that larvae eat approximately twice the amount of leaf material as

they excrete (frass dry weight was approx. 220 mg over the fifth instar in our long-term growth experiments), and that the amount of alkaloid in a 15-mg (dry weight) larva just before pupation is about 20% of the level in its food (e.g., 0.1 and 0.5% dry weight, respectively) (Figure 3), the quantity of quinolizidine alkaloids in the last instar was estimated to be only about 0.5–1% of the amount ingested (15 μg/2.2 mg). (A calculation based on the assumption that larvae eat twice their body weight in foliage per day gave the same estimate.) The concentration of alkaloids in larvae, and the amounts excreted, were strongly correlated with the amounts ingested. These data, taken together, suggest that sequestration was passive. On the other hand, there are presumably mechanisms for concentrating these chemicals in the cuticle. It seems likely that the concentration of alkaloids on or near the surface of the larvae, rather than a more diffuse distribution of these host-derived compounds throughout the body, provides an efficient use of them in deterring predators.

These results contrast with those of studies on other species of Lepidoptera which store host plant-derived pyrrolizidine alkaloids. For example, larvae of two arctiid moths, *Arctia caja* and *Tyria jacobaeae*, accumulate alkaloids derived from the host, *Senecio jacobaea* (Compositae), to levels far in excess of plant concentrations. In the case of *T. jacobaeae* at least, there is differential uptake of pyrrolizidine alkaloids from the food (Aplin and Rothschild, 1972). As in other aposematic species in the family Arctiidae, the alkaloids are kept through the pupal stage and have defensive and reproductive roles in the adults (e.g., Rothschild et al., 1979). A large number of insect species from different orders and families appear to use pyrrolizidine alkaloids in both defense and reproduction (e.g., Schneider, 1987).

Less is known about quinolizidine alkaloid use by insects; it appears to be less common. This may be a reflection of the limited distribution of these alkaloids in general or the limited study of insects feeding on them. Studies by Wink and his coworkers (Wink et al., 1982; Wink and Römer, 1986; Wink, 1987) and Smith (1966) indicate that generalist aphids are deterred by quinolizidine alkaloids, while some specialists are stimulated to feed by them. In addition, a specialist aphid on lupines, *Macrosiphon albifrons*, appears to be chemically defended from predators by sequestered quinolizidine alkaloids (Wink and Römer, 1986). There are numerous accounts of deterrent or detrimental effects of different lupine alkaloids on generalist folivores, while specialists are unaffected (Kinghorn and Balandrin, 1984, and references therein; Wink, 1987; Johnson and Bentley, 1988). In the case of *U. reversalis* there appear to be some positive effects on growth, but it is not known whether this is due simply to increased feeding. In preliminary experiments, larvae were found to feed on standard artificial diets, but fed more readily when *G. monspessulana* powder was added, further supporting the evidence that alkaloids are phagostimulatory (although other plant components may also have been responsible). The larvae,

which have many typical features of aposematic insects (Bernays and Montllor, 1989), are avoided by wasps and ants in choice tests which include a palatable alternative prey (Bernays and Cornelius, 1989). Therefore, the functional value of host-derived alkaloids to these insects is likely to be related to defenses against predators.

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PLANT GROWTH REGULATORY ACTIVITIES OF ARTEMISININ AND ITS RELATED COMPOUNDS

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Abstract—Artemisinin, a sesquiterpene lactone produced in the leaves of *Artemisia annua*, was evaluated for its phytotoxicity in mono- and dicotyledonous plants. Artemisinin inhibited seed germination, seedling growth, and root induction in all species tested. The concentration of artemisinin required for 50% inhibition of *Lemna minor* growth was 5 μM. Inhibitory plant responses appeared to require the endoperoxide moiety of this compound since similar chemicals without endoperoxide, deoxyartemisinin, arteannuic acid, and arteannuin B, were less phytotoxic. In *L. minor*, artemisinin and arteannuic acid caused the leakage of proteins into the growth medium, suggesting the site of activity was at the plant cell membrane.

Key Words—Allelopathy, seed germination, *Lemna* spp., *Artemisia annua*.

INTRODUCTION

Artemisinin, a sesquiterpene lactone containing an endoperoxide, is an antimalarial agent produced in the leaf tissue of *Artemisia annua* L. (China Cooperative Research Group, 1982; Klayman et al., 1984). Its medicinal value has been reviewed (Klayman, 1985; Luo and Shen, 1987). Recently, Duke et al. (1987) reported on the selective phytotoxicity of artemisinin.

Drugs, insecticides, and many other useful chemicals have been isolated from various species of plants in the genus *Artemisia* (Sherif et al., 1987). There are more than 2000 sesquiterpene lactones that have been isolated from various plants and fungi (Fischer, 1986; Stevens, 1984). This group of compounds pos-

sesses a variety of biological activitites; some are antiparasitic, antineoplastic, antimicrobial, or schistosomicidal with the potential to be used as drugs for man and animals. Others are insect antifeedants or growth regulators and have potential for use in agriculture in the future (Duke et al., 1987; Fischer, 1986; Stevens, 1984; Thompson, 1985).

Fischer (1986) reviewed the research on the allelopathic and plant growth regulatory activity of mono- and sesquiterpenes. He reported that although many of these compounds have been tested for seed germination and plant growth activity, much work is needed to determine the structural moieties essential for bioactivity and the mechanisms of action. We began our investigation of the effects of some secondary metabolites of *A. annua* on other plants independently from Duke et al. (1987), and we evaluated their effects with some unique test species. Here we report not only the effects of artemisinin (in China artemisinin is known as qing-hao-su), but also its related compounds on both terrestrial and aquatic plants.

METHODS AND MATERIALS

Chemicals Used. Pure compounds (Figure 1) used in this investigation were artemisinin, arteannuic acid, arteannuin B, deoxyartemisinin, artesunic acid, sodium artelinate, and santonin. Santonin was purchased from Sigma Chemical Co. Other compounds were isolated and/or synthesized by Drs. D.L. Klayman, N. Acton and A.J. Lin of Walter Reed Army Institute of Research, Washington, D.C. Test solutions were freshly prepared by first dissolving the compounds in 100% methanol as stock solutions. Final concentrations were obtained by dilution into the growth medium of the test species. Controls received amounts of methanol equal to the highest concentration in the test solutions.

Seed Germination. Seeds used in this study were carrot (*Daucus carrota*), lettuce (*Lactuca sativa*), purchased from the Carolina Biological Supply; giant foxtail (*Setaria faberi*), barnyardgrass (*Echinochloa crus-galli*), and crabgrass (*Digitaria sanguinalis*) collected from the field. Fifty seeds were placed on Whatman No. 3 filter paper moistened with 3 ml of test solution in a 9-cm Petri dish. All the Petri dishes containing seeds and test solution were maintained in a germinator at 25°C with 90% humidity and a 12-hr dark–light (18.8 $\mu E/m^2/$ sec) cycle for seven days. At harvest, germination was scored and seedling fresh and dry weights determined.

Root Induction. Seedlings of bush bean (*Phasoleus vulgaris*) and mung bean (*Phaseolus aureus*) were used for root induction experiments. Bean seeds were germinated and seedlings grown in the greenhouse for 7–10 days in pots containing a sterile soil mix (2:2:1 sand–loam–peat on a volume basis). The

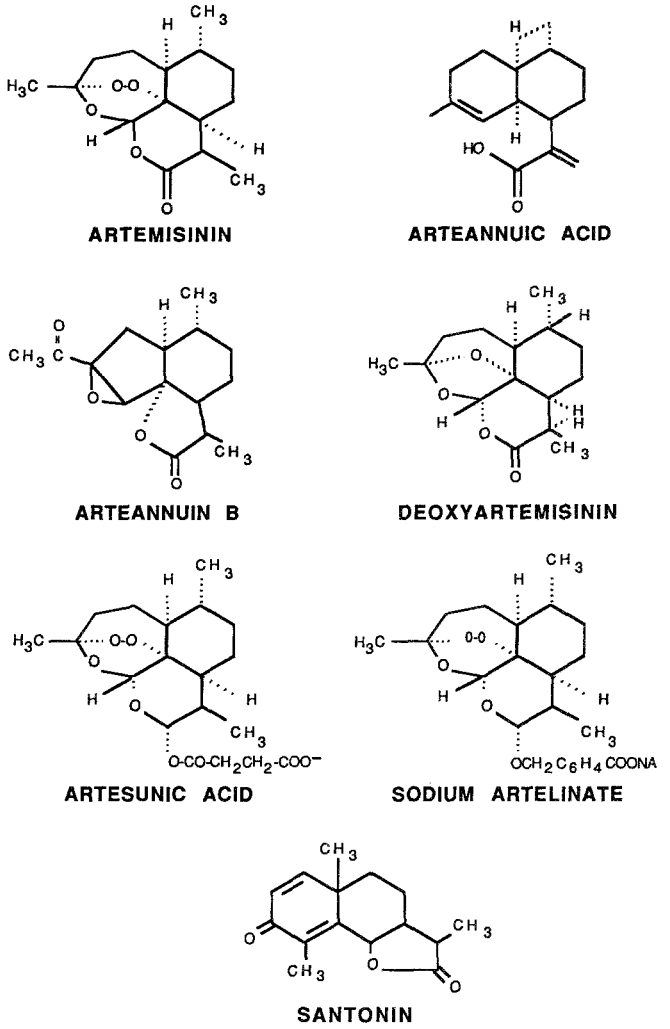

FIG. 1. Structures of artemisinin and its related compounds.

7- to 10-day-old seedlings were cut at the soil surface, washed, blot-dried with paper towels, and introduced into 945-ml Mason jars (bush bean) or 13 × 100-mm test tubes (mung bean) containing the test compound at a concentration of 1 mg/100 ml solution. The Mason jars containing 150 ml distilled water and test tubes containing 6 ml distilled water were covered with aluminum foil and sterilized by autoclaving before adding the chemicals.

Lemna *Assay.* The aquatic duckweeds, *Lemna minor* and *Lemna obscura*,

were grown in cotton-plugged Erlenmeyer flasks (125 ml) containing 50 ml of E medium (Cleland, 1979), which was sterilized by autoclaving at 15 psi for 15 min. The cultures were grown in an environmental chamber at 28°C under constant light (236 $\mu E/m^2/sec$) provided by fluorescent and incandescent bulbs for seven days. For bioassay, duckweed at the three- or four-frond stage were transferred to a 24-well tissue culture plate containing 1.5 ml E medium (without sucrose and tartaric acid) amended with the appropriate test compound in each well. After incubation under the same conditions for seven days, the growth and pigment contents were determined according to the methods and procedures described by Einhellig et al. (1985) and Leather and Einhellig (1985). Protein in the spent medium was colorimetrically determined by the method of Bradford (1976).

Germination and root induction experiments required large quantities of compounds and were not repeated for statistical analyses. Data from experiments with *Lemna* were analyzed by Student's *t* test and significance determined by nonoverlapping confidence levels at $P = 0.05$.

RESULTS AND DISCUSSION

Duke et al. (1987) investigated the effect of artemisinin on seed germination and seedling growth and reported that artemisinin was selectively toxic to plants. However, the effect of arteannuic acid and arteannuin B on seed germination and root and seedling growth was not clear. Arteannuic acid and arteannuin B are known to be precursors of artemisinin (Akhila et al., 1987; El-Feraly et al., 1986). Thus, it was of interest to determine the biological activity and the structural relationships of the biologically active chemicals.

Artemisinin inhibited lettuce seed germination and seedling growth as determined by fresh and dry weight (Table 1). Arteannuic acid exerted no effect on seed germination, but appeared to increase slightly the seedling dry weight. Arteannuin B did not affect lettuce seed germination or seedling growth.

Artemisinin is only slightly soluble in water. Therefore, artesunic acid, a modified compound that is one of the forms of artemisinin used in treating malaria, was used to investigate its relative biological activity. The results shown in Table 2 indicate that artesunic acid is similar to artemisinin in that it inhibited seedling growth but was less effective on seed germination. The inhibition of lettuce seed germination and seedling growth by artemisinin (Table 1) was greater than that of artesunic acid (Table 2). This difference may be attributed to differences in species sensitivity, the lipophilic nature of the compound, or because artesunic acid is not stable in water; although artemisinin is less soluble, it is stable in water (Klayman, 1985).

During our investigation on seed germination and seedling growth of var-

TABLE 1. EFFECTS OF SECONDARY METABOLITES OF *Artemisia annua* ON GERMINATION AND SEEDLING GROWTH OF LETTUCE

Treatment	Concentration (μg/dish)	Germination[a] (%)	Fresh wt.[b] (mg/dish)	Dry wt. (mg/dish)
Control	0	100.0	472.0 ± 0.5	50.4 ± 0.2
Artemisinin	50	55.2	171.2 ± 15.7	29.6 ± 0.7
	100	59.4	167.2 ± 28.9	27.2 ± 2.2
	200	42.7	103.5 ± 25.6	20.9 ± 3.1
Arteannuic acid	50	97.9	495.5 ± 40.3	61.5 ± 2.7
	100	98.9	460.6 ± 30.9	53.6 ± 2.3
	200	95.8	453.2 ± 27.7	55.8 ± 2.5
Arteannuin B	50	91.7	457.5 ± 13.9	51.3 ± 3.8
	100	88.5	385.6 ± 25.2	46.6 ± 2.4
	200	87.5	418.0 ± 21.3	51.8 ± 9.8

[a] Each Petri dish contained 50 seeds and was replicated four times. As percent of controls.
[b] Values are ± standard error of the means.

TABLE 2. EFFECT OF ARTESUNIC ACID ON SEED GERMINATION AND SEEDLING GROWTH

Plant	Concentration (μg/dish)	Germination[a] (%)	Fresh wt.[b] (mg/dish)
Barnyardgrass	0	100.0	325.5 ± 31.9
	50	98.2	242.5 ± 17.5
	100	87.3	216.0 ± 69.6
	200	78.2	170.3 ± 46.3
Carrot	0	100.0	296.4 ± 9.8
	50	100.0	182.4 ± 2.2
	100	98.6	163.9 ± 33.0
	200	90.0	115.0 ± 26.0
Crabgrass	0	100.0	115.7 ± 12.3
	50	96.8	68.5 ± 59.2
	100	95.8	49.3 ± 3.5
	200	87.4	56.3 ± 6.0
Green foxtail	0	100.0	116.0 ± 23.2
	50	117.3	126.4 ± 1.3
	100	107.7	65.8 ± 7.3
	200	57.7	48.1 ± 3.3

[a] As percent of controls.
[b] Values are ± standard error of the means.

ious plants (Tables 1 and 2), we observed that radicles of the germinating seeds were significantly shorter in the higher concentrations of artemisinin and artesunic acid. Bush bean seedlings 7–10 days old were used to investigate the effects of artemisinin, arteannuic acid, and arteannuin B on root induction. The results are shown in Table 3. Both arteannuin B- and arteannuic acid-treated seedling cuttings showed an increase in number of roots formed; however, the mean length of roots was less than the controls (32.2 and 40.2%, respectively). It is interesting to note that artemisinin completely inhibited the root formation in bush bean seedlings, which resulted in less shoot growth.

In order to elucidate the relationship of chemical structure to biological activity, it was necessary to use a test system that required minimal amounts of material since most compounds shown in Figure 1 were not commercially available and were difficult to obtain. We found that mung bean seedlings were smaller and required less compound per test. When mung bean seedlings were used, the results were similar and artemisinin completely inhibited root induction. Both arteannuic acid and arteannuin B stimulated root induction in bush and mung bean seedling cuttings (Tables 3 and 4). The structure–activity relationships of artemisinin and its derivatives have been reviewed (Luo and Shen, 1987). From the data in Table 4, a close relationship of chemical structure and biological activity was clearly demonstrated. Both deoxyartemisinin and santonin are sesquiterpenes similar to arteannuic acid and arteannuin B; that is, they do not contain an endoperoxide moiety. These compounds stimulated root induction. Artesunic acid and sodium artelinate are similar to artemisinin and contain an endoperoxide moiety that exerted the same magnitude of inhibitory effect on root induction in bean seedling cuttings.

Leather and Einhellig (1986) reported that both *L. minor* and *L. obscura* are very sensitive to allelochemicals. With these systems, we observed that as little as 10 μM of artemisinin significantly inhibited production of fronds in both species and chlorophyll synthesis in *L. minor* and anthocyanin content in

TABLE 3. EFFECT OF SECONDARY METABOLITES OF *Artemisia annua* ON ROOT INDUCTION AND SHOOT GROWTH OF BLACK VALENTINE BUSH BEAN[a]

Treatment	Root		Shoot growth length (mm)
	No.	Length (mm)	
Control	5.8 ± 2	25.7 ± 10	57.8 ± 12
Arteannuin B	10.2 ± 4	17.4 ± 3	56.0 ± 13
Arteannuic acid	17.2 ± 3	15.4 ± 7	54.0 ± 12
Artemisinin	0.0	0.0	42.0 ± 24

[a]Concentration was 1 mg/100 ml solution, values are ± standard error of the means.

TABLE 4. EFFECTS OF ARTEMISININ AND RELATED COMPOUNDS ON ROOT INDUCTION IN MUNG BEAN[a]

Chemical used	Amount (μl)[b]	Average No. of roots[c]			Normalized percentage
		Long	Medium	Short	
Control (water)		5.0	1.7	2.7	100.0
Methanol	20	3.7	3.3	1.3	88.3
Arteannuic acid	10	6.0	6.0	7.0	202.1
	20	5.5	1.0	2.0	90.4
Arteannuin B	10	4.7	3.3	6.3	152.1
	20	2.5	8.5	1.0	127.7
Deoxyartemisinin	10	3.3	6.3	3.7	141.5
	20	4.0	7.5	4.0	164.9
Santonin	10	5.0	6.7	0.3	127.7
	20	3.5	8.0	0.0	122.3
Artemisinin	10	0.0	0.0	0.0	0.0
	20	0.0	0.0	0.0	0.0
Artesunic acid	20	0.0	0.0	0.0	0.0
Sodium artelinate	20	0.0	0.0	0.0	0.0

[a] Average of triplicate samples.
[b] Volume of a solution containing 1 mg chemical/100 ml HOH.
[c] Long roots > 2 cm; medium roots 0.5–1.9 cm; short roots < 0.5 cm.

L. obscura (Table 5). In *L. minor*, 5 μM artemisinin inhibited frond production 50% (data not shown). Duke et al. (1987) reported a similar concentration range of activity for artemisinin. In these experiments it was noted that arteannuic acid exerted a severe leaking effect on *L. minor* and *L. obscura*. However, artemisinin appeared to affect the plasma membrane only, since at lower concentrations some chlorophylls (in the chloroplasts) and anthocyanin (within the tonoplasts) were retained.

The effects of artemisinin and arteannuic acid on the membrane were further supported by the data shown in Table 6. Protein in the spent medium, taken at one- to eight-day intervals, showed that the effects of arteannuic acid can be observed after 24 hr. At the first time interval, the protein content of the spent medium containing 100 μM of arteannuic acid was fivefold greater than the controls. Among the arteannuic acid-treated samples, the protein content of the medium increased more than 20 times that of the controls. The amount of protein in the spent medium of artemisinin-treated *Lemna* increased slightly after two to three days. At the end of the experiment, there was a 12-fold increase in protein. During this period of investigation (eight days), there was no increase in the number of *Lemna* fronds in all the wells containing 100 μM of artemisinin or arteannuic acid. The total extracellular protein content in the control wells

TABLE 5. EFFECTS OF ARTEANNUIC ACID, ARTEANNUIN B, AND ARTEMISININ ON *Lemna* SPP.[a]

Compound	Conc. (μM)	*L. minor* No. fronds	*L. minor* Chlorophyll (μg/frond)	*L. obsura* No. fronds	*L. obsura* Anthocyanin (μg/frond)
Arteannuic acid	500	4 a	0.00 a	3 a	0.04 a
	250	4 a	0.00 a	4 a	0.04 a
	100	4 a	0.00 a	4 a	0.08 ab
	50	10 abc	0.15 a	8 abc	0.06 a
	10	10 abc	0.18 a	10 cde	0.09 abc
Arteannuin B	500	4 a	0.00 a	4 a	0.08 ab
	250	14c	0.40 b	9 bcd	0.08 ab
	100	33 d	0.56 bcd	13 ef	0.15 d
	50	33 d	0.68 cde	16 f	0.12 bcd
	10	38 d	0.74 de	26 g	0.17 d
Artemisinin	500	4 a	0.00 a	4 a	0.13 bcd
	250	4 a	0.00 a	4 a	0.14 bcd
	100	4 a	0.87 e	5 ab	0.12 bcd
	50	5 a	0.57 bcd	6 abc	0.09 abc
	10	12 bc	0.51 bc	12 def	0.13 bcd
Control		51 e	0.83 e	30 h	0.28 e

[a] Means in a column having the same letters are not significantly different as determined by nonoverlapping confidence limits at the 0.05 level.

TABLE 6. PROTEIN CONTENT OF SPENT MEDIUM AFTER GROWTH OF *Lemna minor*[a]

Days in culture	Control (μg/frond)	Protein content in medium containing: Arteannuic acid (μg/frond)	Protein content in medium containing: Artemisinin (μg/frond)
1	5.8 a	25.8 b	6.7 a
2	4.8 a	31.6 c	11.4 b
3	2.8 a	30.9 c	7.8 b
5	1.1 a	33.3 c	14.1 b
7	1.8 a	42.4 c	16.2 b
8	1.9 a	37.4 c	24.0 b

[a] The concentration of arteannuic acid and artemisinin was 100 μM. Values in a row for culture day having the same letters are not significantly different as determined by nonoverlapping confidence limits at the 0.05 level. On the eighth day the number of fronds was 30–35 in the controls, but only four in both arteannuic acid- and artemisinin-treated wells.

increased only three-fold, whereas the number of fronds increased 8- to 13-fold. Duke et al. (1987) suggested that the mechanism of plant growth inhibition by artemisinin was not the disruption of mitosis, amino acid synthesis, or the inhibition of respiration. The apparent effects of artemisinin on the plasma membrane we observed in *Lemna* still does not explain the inhibition of germination and root induction found in this research and previously reported by Duke et al. (1987).

Results obtained from this study suggest that artemisinin may be phytotoxic not only to terrestrial plants but that it also possesses significant inhibitory effects against aquatic weeds such as *L. minor* and *L. obscura*. It is clear that artemisinin and those related compounds that possess an endoperoxide moiety showed inhibitory effects on root induction in bean plants. However, arteannuic acid and arteannuin B seem to stimulate more short root formation on bush and mung bean seedling cuttings. These two sesquiterpene compounds may serve as root-inducing agents and warrant further exploration.

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CHANGE IN MANDIBULAR AND MESOSOMAL GLAND CONTENTS OF MALE *Xylocopa micans* (HYMENOPTERA: ANTHOPHORIDAE) ASSOCIATED WITH MATING SYSTEM[1]

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Abstract—Males of *Xylocopa micans* employ two mating systems in south-central Texas. They defend territories around flowering wisteria and redbud in March and April, and they maintain nonresource-based or landmark territories in July and August. Mandibular and mesosomal gland contents (analyzed by GC-MS) are different in bees employing the two mating systems. Mandibular glands contain only straight-chain hydrocarbons in bees defending floral resources, but include ethyl oleate in bees defending landmark territories. Mesosomal glands contain saturated, mono- and diunsaturated straight-chain hydrocarbons and methyl and ethyl esters of long-chain fatty acids. The major ethyl ester, ethyl oleate, comprises only 1.1% of mesosomal gland contents in bees defending floral resources but comprises 39.7% of gland content of bees defending landmark territories. These findings are discussed relative to the proposed sex pheromone function of *Xylocopa* gland secretions.

Key Words—*Xylocopa micans*, Hymenoptera, Anthophoridae, mesosomal glands, mandibular glands, hydrocarbons, fatty acid esters, territoriality, mating system.

INTRODUCTION

Male carpenter bees in the genus *Xylocopa* exhibit a variety of mating systems. Marshall and Alcock (1981) divided mating systems of *Xylocopa* into four categories: (1) female defense polygyny (male territoriality at the female emer-

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gence site), (2) resource defense polygyny (male territoriality at flowers or female nesting site), (3) lek polygyny (male territoriality at nonflowering plants and landmarks), and (4) nonterritorial patrolling. The majority of *Xylocopa* species maintains territories of one type or another (e.g., Barrows, 1983; Frankie et al., 1979; Gerling and Hermann, 1978; Gerling et al., 1983: Velthius and de Camargo, 1975a; Velthius and Gerling, 1980).

Chemicals from several glands of male *Xylocopa* have been implicated in maintaining territories and attracting females (Gerling et al., 1989). Hefetz (1983) observed that male *X. sulcatipes* Maa marked plants with mandibular secretion, containing guaiacol, *p*-cresol, and vanillin, that functions as a sex pheromone. Velthius and de Camargo (1975b) suggested that male *X. hirsutissima* Maidl spread mandibular secretions over the venter of the abdomen to attract females to their territory.

Dorsal mesosomal glands, described by Vinson et al. (1986), also have been proposed to contain a sex pheromone in some *Xylocopa* species. Two Costa Rican species, *X. gualanensis* Cockerell and *X. fimbriata* F., appear to smear mesosomal secretions on their legs, which they then use to mark vegetation (Vinson and Frankie, 1990). Andersen et al. (1988) proposed a sex pheromone role for terpenoids identified from *X. varipuncta* Patton and fatty acid esters and alcohols identified from *X. micheneri* (Hurd) mesosomal glands.

Xylocopa (*Schonherria*) *micans* Lepeletier occurs commonly in south-central Texas where Frankie et al. (1979) observed males defending nonresource-based or landmark territories (lek polygyny) in late July. In this study, we describe another mating system, that of floral resource defense (resource defense polygyny), exhibited in early spring. We isolated and identified secretions from both mandibular and mesosomal glands and compared their contents in bees displaying different mating systems.

METHODS AND MATERIALS

Behavioral Observations. Floral defense behavior of male *X. micans* was observed over a period of three years. Males were studied on wisteria (*Wisteria macrostachys*) during March 1987 and April 1989, and on redbud (*Cercis canadensis*) in early March 1988 within College Station city limits, Brazos County, Texas. Males also were observed during late March 1989 on wisteria in Needville, Brazoria County, Texas.

Isolation of Glandular Material. Male *X. micans* were collected while they defended nonresource-based territories along unpaved roads in West Columbia, Brazoria County, Texas, during August 1988 [collection area described by Frankie et al. (1979)]. Males exhibiting floral resource defense were collected while they defended flowering wisteria bushes in Needville, Brazoria County,

Texas (22 km northwest of West Columbia) in late March 1989. Bees were placed individually in glass vials and transported on ice to the laboratory in College Station where they were kept at 4°C until they were dissected (within one week).

Bee heads were removed ($N = 20$ for August 1988 and $N = 18$ for March 1989 collection), and pairs of mandibles and mandibular glands were dissected out and pooled by collection date in hexane (HPLC grade, Fisher). Mesosomal glands were dissected out with fine forceps, keeping gland tubules attached to the overlying cuticle to prevent loss of glandular material. Mesosomal glands and the attached cuticle from individual bees were placed in glass vials containing 30 μl hexane (with 0.1 μg/μl tetradecane as an internal standard). After 10 min, 20 μl of solvent were removed and placed in autosampler vials for individual analysis. A sample of gland tubules only was prepared to determine whether cuticular components contaminated mesosomal gland samples.

Identification of Gland Components. Gland extracts were analyzed using a Hewlett Packard 5890 gas chromatograph (GC) equipped with flame ionization detector and Hewlett Packard 7673A automatic sampler. Volatile components were separated on a 25-m × 0.22-mm ID BP1 fused silica capillary column with He head pressure of 20 psi, and temperature programmed at 60°C for 1 min to 280°C at 10°C/min. Mass spectral data were obtained using a HP 5890A GC/HP 5970B Mass Selective Detector with 30-m × 0.22-mm ID BP1 capillary column. Gland components were identified by comparison of recorded mass spectra with those of a spectral library and by comparison of GC retention times with those of purchased chemicals (Sigma Chemical Co.).

RESULTS

Behavioral Observations. During March and April, 1987 through 1989, male *X. micans* were observed defending territories around flowering wisteria and redbud. During early spring in south-central and southeast Texas these species are abundant floral resources that are patchily distributed and on which female *X. micans* forage in large numbers. Males typically defended an entire tree and slowly flew 1–4 m above the ground (depending on tree height) while facing towards the tree. Males vigorously chased intruding conspecific males and other insects such as honeybees and flies. In the case of a conspecific intruder, the chase often escalated into a "swirling" flight above the crown of the tree.

When a male *X. micans* encountered a female on a blossom, he hovered ca. 40 cm from her. On two occasions a male was observed to dart at the female and a distinct "clack" was heard. The male left the female immediately, probably without mating.

Identification of Gland Components. Extracts of mandibular glands and mandibles were compared with extracts of abdominal cuticle and were found to contain the same chemicals in similar proportions in bees defending floral resources (Figure 1A). However, in bees defending a landmark territory, an additional peak was present (Figure 1B). GC retention time and MS fragmentation pattern were consistent with assignment of this peak as ethyl oleate. Assignment was confirmed by GC and GC-MS analysis of a sample of authentic ethyl oleate.

Gas chromatographic analysis of extracts of isolated mesosomal glands and of mesosomal glands with associated cuticle produced similar chromatograms with components in the same ratios. Therefore, analysis of individual bees was performed using extracts of mesosomal glands with attached cuticle so that glandular material was not lost during dissection.

Mesosomal glands contained mono- and diunsaturated and saturated straight-chain hydocarbons from 17 to 29 carbons in length and fatty acid methyl and ethyl esters (Figure 2). Average amounts of chemicals in the glands and

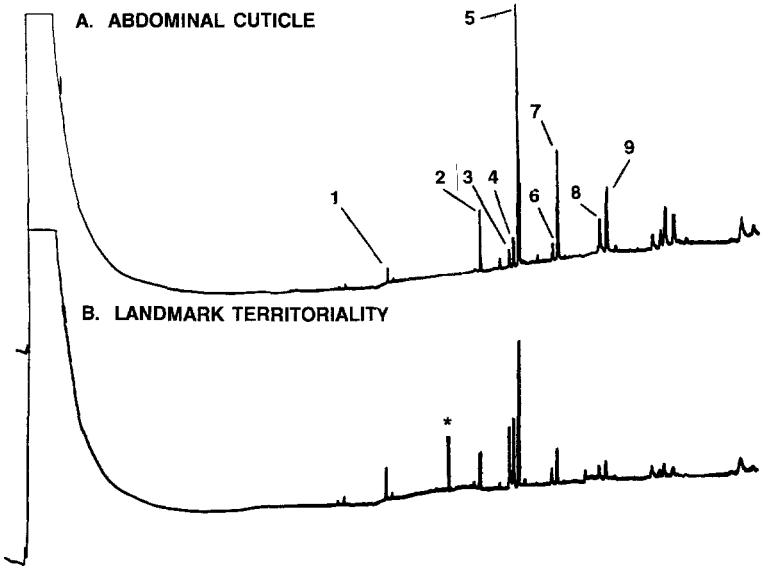

FIG. 1. Comparison of GC chromatograms of hexane extracts of *X. micans*: (A) abdominal cuticle, and (B) mandibles and mandibular glands from males displaying landmark territoriality. Peak 1 = nonadecene, 2 = tricosane, 3 = pentacosadiene, 4 = pentacosene, 5 = pentacosane, 6 = heptacosene, 7 = heptacosane, 8 = nonacosene, and 9 = nonacosane. Peak marked with an asterisk identified as ethyl oleate.

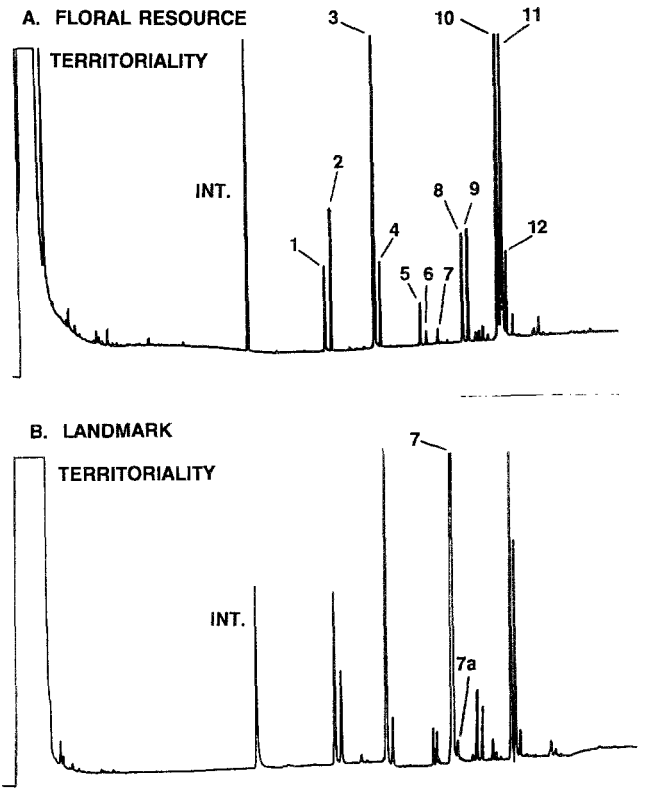

FIG. 2. Comparison of GC chromatograms of hexane extract of mesosomal gland of *X. micans* male displaying floral resource territoriality and gland of a male displaying landmark territoriality. Peaks 1–12 are identified in Table 1. INT. = 0.1 μg tetradecane internal standard and 7a = ethyl stearate.

their percent contribution to total gland content were calculated for components comprising over 0.5% of the gland total (Table 1).

Although there is much interindividual variation in amounts of chemical present in mesosomal glands (Table 1), there is a striking difference in quantities of ethyl oleate in bees using different mating systems. Nonadecene, pentacosadiene, and pentacosene are the most abundant chemicals in glands of bees exhibiting floral resource defense; ethyl oleate comprises only 1.1% of total gland content in these bees. However, in bees maintaining landmark territories, ethyl oleate is the most abundant component and accounts for 39.7% of total gland content. Bees defending floral resources and defending landmark territories have similar total quantities of mesosomal gland secretion (Table 1).

TABLE 1. COMPONENTS OF MESOSOMAL GLANDS OF MALE *X. micans* EXHIBITING FLORAL TERRITORIALITY DURING MARCH AND APRIL (N = 15) AND LANDMARK TERRITORIALITY DURING JULY AND AUGUST (N = 18) IN SOUTH-CENTRAL TEXAS

		Floral resource territoriality			Landmark territoriality		
Peak	Compound	Mean amount (ng)	SD	%	Mean amount (ng)	SD	%
1	Heptadecene	61.5	31.5	3.4	41.0	25.4	2.8
2	Heptadecane	68.8	39.6	3.8	24.6	12.9	1.7
3	Nonadecene	365.7	187.9	20.1	251.7	142.9	17.2
4	Nonadecane	37.3	24.4	2.0	10.0	5.8	0.68
5	Heneicosene	17.5	9.6	0.96	9.5	5.9	0.65
6	Methyl oleate	6.6	4.0	0.36	13.0	12.4	0.89
7	Ethyl oleate	19.9	42.8	1.1	580.6	270.5	39.7
8	Tricosene	58.4	37.3	3.2	24.5	18.0	1.7
9	Tricosane	72.7	52.5	4.0	14.3	13.2	0.98
10	Pentacosadiene	672.4	417.4	37.0	377.8	219.5	25.8
11	Pentacosene	388.1	208.0	21.3	105.5	72.9	7.2
12	Pentacosane	49.0	30.8	2.7	9.6	8.9	0.66
	total	1818	1505	100	1462	687	100

DISCUSSION

Many male *Xylocopa* bees establish and maintain territories for the purpose of mating (Alcock, 1980); however, the location and timing of territorial behavior varies within this large genus. Moreover, mating strategies may vary within a species and even within an individual. *Xylocopa californica arizonensis* Cresson exhibits nonterritorial patrolling (Hurd and Linsley, 1975) and also territoriality at the female nest (Hurd, 1958; O'Brien and O'Brien, 1966). *Xylocopa sulcatipes* males may defend territories near nesting sites or at flowering plants (Velthius and Gerling, 1980). *Xylocopa virginica virginica* L. may have as many as five mating systems including territoriality at nest sites, at food resources, and at landmarks (Barrows, 1983). In some species, males may exhibit all mating strategies, whereas in other cases males may be specialized to employ only one type of strategy (Alcock et al., 1978). The present study indicates that *X. micans* employs at least two mating systems, only one of which relies on release of a sex pheromone.

Various authors have indicated a sex pheromone role for mandibular gland secretions. Although several chemicals have been identified, behavioral bioassays to confirm the role of these isolated chemicals have rarely been performed. Wheeler et al. (1976) identified a lactone as the major component of the man-

dibular secretion of *X. hirsutissima*. This bee performs lek polygyny and hovers around bushes on mountain tops. Females are supposedly attracted by mandibular secretions to these territories. Hefetz (1983) identified mandibular gland contents of *X. sulcatipes* as guaiacol, vanillin, *p*-cresol, and a series of homologous hydrocarbons. Behavioral bioassays indicated that vanillin serves to attract females to the territory and guaiacol elicits aggression in male bees.

Studies by Andersen et al. (1988) and Williams et al. (1987) have shown that mesosomal glands of Arizonan and Costa Rican *Xylocopa* bees contain either terpenoids (*X. gualanensis* and *X. varipuncta*), fatty acid derivatives (*X. micheneri*), or chemicals from several biosynthetic pathways (*X. fimbriata*). *Xylocopa micans* mesosomal glands contain only chemicals produced via the fatty acid synthetase pathway, and the main component, ethyl oleate, is similar to isopropyl oleate, the main component of *X. micheneri* gland secretions.

Ethyl oleate is clearly the major component of mandibular and mesosomal gland secretions of male *X. micans*, but the question remains as to the significance of the series of saturated and monounsaturated hydrocarbons. These same chemicals are found in the abdominal cuticle, but in lesser amounts, and could conceivably originate from mesosomal gland contents spreading over the lipophilic cuticle. However, the same chemicals are found in quantity in Dufour's glands of female *X. micans* and *X. virginica texana* Cresson (Williams et al., 1983). Their presence in female *X. micans* also may indicate that these hydrocarbons are simply constituents of untanned cuticle (e.g., Dufour's gland and the tubules of the mesosomal gland). It is more likely, however, that these hydrocarbons are gland contents with some as yet unknown function.

Ethyl esters are also common in the Dufour's gland of female *X. virginica texana* (Williams et al., 1983) and in several other bee species (Hefetz, 1987). When applied to passion flowers, these esters repel conspecific female *X. virginica texana* for a period of 10 min (Vinson et al., 1978). It is reasonable that both male and female *X. micans* possess the biosynthetic capability to produce ethyl esters and use different ethyl esters to convey their sex-specific messages.

In summary, it appears that male *X. micans* use two mating systems under different situations in south-central Texas. Wisteria and redbud flower early in the spring and are rich, patchily located nectar sources for foraging females. Males take advantage of the clumped distribution of females and defend territories around these floral resources. At this stage, no sex attractant would be necessary as females are already attracted to the nectar source, and correspondingly, we see little ethyl oleate in mandibular and mesosomal glands of males employing this tactic. As the environmental conditions change, either individual males change their mate location strategy or a second genotype or a second generation of male appears that uses a different system. At this stage, males choose prominent landmarks (Frankie et al., 1979) and establish territories in areas that contain no resource needed by the female. Mesosomal and mandib-

ular glands of males now contain ethyl oleate as their major constituent. We hypothesize that ethyl oleate acts as an attractant and may demonstrate male fitness to females that fly through the territory.

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ELECTROPHYSIOLOGICAL AND CHEMICAL ANALYSIS OF SEX PHEROMONE COMMUNICATION SYSTEM OF THE MOTTLED UMBER, *Erannis defoliaria* (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—(*Z*,*Z*,*Z*)-3,6,9-nonadecatriene (*Z*3,*Z*6,*Z*9–19Hy) and (*Z*,*Z*)-3,9-*cis*-6,7-epoxy-nonadecadiene (*Z*3,*Z*9-*cis*-6,7-epo-19Hy) were identified in pheromone gland extracts from female *Erannis defoliaria*. The two components were found in a 1:3 ratio, with the main component, *Z*3,*Z*9-*cis*-6,7-epo-19Hy present at an amount of about 1.5 ng per female. The components were identified by means of gas chromatography–mass spectrometry, gas chromatography–electroantennography and gas chromatography–single sensillum recordings. Single sensillum measurements on the male antenna showed two physiological types of sensilla. One type was characterized by a large spike amplitude cell responding to *Z*3,*Z*9-*cis*-6,7-epoxy-19Hy and a small spike amplitude cell responding to *Z*3,*Z*6,*Z*9–19Hy. A second type responded only with a large spike amplitude cell to the epoxide, and this cell was inhibited by the triene. Of the two pheromone components, the epoxide gave the higher response in the EAG tests. Preliminary field tests support the identification of the pheromone components. The epoxide was also found to be present in the extract of the pheromone gland of *Colotois pennaria*, and males of *C. pennaria* and *Agriopis marginaria* were trapped by the mixture of the identified compounds.

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Key Words—*Erannis defoliaria* (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene, (*Z*,*Z*)-3,9-*cis*-6,7-epoxy-nonadecadiene, sex pheromone, identification, electroantennogram, single sensillum, peripheral inhibitor, *Agriopis marginaria*, *Colotois pennaria*, Lepidoptera, Geometridae.

INTRODUCTION

Adults of the mottled umber, *Erannis defoliaria* Clerck (Lepidoptera: Geometridae) are active at low temperatures in the late autumn. The female of this species, as in several other geometrids, is wingless. The female crawls up a tree trunk to emit the pheromone and attract conspecific males (Tvermyr, 1969). *E. defoliaria* is commonly found in most parts of Europe, where its larvae feed on foliage of almost any deciduous tree or shrub. It is particularly common on oak, birch, and hawthorn (Escherich, 1931), but sometimes attacks orchards, where it may cause considerable damage (Carter, 1982).

Sex attractants for male moths have been documented in numerous geometrids, but an actual pheromone has been described for only about 10 species (Arn et al., 1986, and references therein). Straight-chain homoconjugated polyenic hydrocarbons with 17 or 19 carbon atoms, and epoxides derived from these, are the compounds most often identified as pheromone components in the Geometridae. Similar compounds have also been identified as pheromone components in Arctiidae and in a few genera of Noctuidae (see Arn et al., 1986).

We initiated the present work because *E. defoliaria* is regarded as an important pest in Northern and Middle Europe (Escherich, 1931; Kovacs, in Balas and Saringer, 1982), and also to expand the limited basic knowledge of geometrid pheromone communication systems.

METHODS AND MATERIALS

Insects. Larvae hatched from overwintering eggs laid by adults collected in Budapest, Hungary, were reared on cherry leaves to pupation. Before the flight period, pupae were carefully removed from the soil of the rearing containers, sexed, and subsequently stored on moistened filter paper in Petri dishes until eclosion. Emerging adults were collected daily and transferred to 1-liter glass jars, where they were supplied with water on cotton wool. During their whole life cycle, insects were kept under outdoor conditions. Insects used in electrophysiological studies in Lund were transferred to Sweden as pupae.

Extracts. Females were observed to establish the time of calling and the presumed optimal time for pheromone gland extraction. To collect the pheromone, the terminal segments of the abdomen of calling unmated females were

excised and extracted in a minimal amount of redistilled hexane for about 10 min.

Gas Chromatography. Extracts were analyzed by gas chromatography (GC) on a Hewlett Packard 5880 gas chromatograph equipped with a flame ionization detector (FID). Hydrogen was used as carrier gas. Two to four microliters of the extract were injected on a capillary column. The samples were run on a nonpolar DB1 column (30 m × 0.25 mm ID) (J&W Scientific, Folsom, California 96830) at an initial temperature of 100°C for 2 min, and a temperature program of 15° C/min up to 240°C, and on a medium polarity DB wax column (30 m × 0.25 mm ID) (J&W) at an initial temperature of 80°C for 2 min, and a program of 10°C/min up to 230°C. On both columns, the equivalent chain lengths compared to a homologous series of straight-chain acetates (8:OAc–17:OAc) were established (Swoboda, 1962).

The amount of pheromone components present in the females was established by comparison to a reference of known concentration.

A HP 5830 GC equipped with an effluent split and a DB1 column allowed simultaneous FID and electroantennographic detection (FID-EAD) (Arn et al., 1975) and simultaneous FID and single sensillum detection (FID-SC) (Wadhams, 1982; Van der Pers and Löfstedt, 1983). Hydrogen was used as carrier gas, and the effluent split ratio was approximately 1:1. The outlet for the EAD was placed in a purified airstream flowing over the antennal preparation at a speed of 0.5 m/sec. The electrophysiological methods were identical to the ones described below.

Mass Spectrometry. For structural determinations a VG 250 S mass spectrometer (70 eV) linked to a HP 5890 gas chromatograph was used. Gas chromatographic separation was achieved on a fused silica DB5 column (30 m × 0.25 mm ID; J&W), maintained for 3 min at an initial temperature of 60°C and then heated to 300°C at a rate of 4°C/min.

Synthesis. (*Z*,*Z*,*Z*)-3,6,9-Nonadecatriene was prepared from linolenic acid and a 10-fold excess of propanoic acid by a mixed Kolbe electrolysis according to known methods (Conner et al., 1980). The crude product was purified by preparative gas chromatography on a 4m × 6-mm-ID column packed with OV-101 on Chromosorb W-AWDMCS at 220°C. The overall chemical purity was >98%.

(*Z*,*Z*,*Z*)-3,6,9-Nonadecatriene was converted to a mixture of the three corresponding monoepoxydienes by oxidation with *m*-chloroperbenzoic acid (Wong et al., 1985). The three isomers could be separated with hexane–diethylether at a ratio of 97:3 on a DB5 column (Merck) (30 m × 0.25 mm ID) maintained at an initial temperature of 60°C for 3 min and then heated to 300°C at a rate of 3°C per minute. (*Z*,*Z*)-3,9-*cis*-6,7-epoxynonadecadiene proved to be the first eluted epoxide, followed by (*Z*,*Z*)-3,6-*cis*-9,10-epoxynonadecadiene and (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene, respectively. NMR spectra

and mass spectra were in accordance with those of the homologous C_{17} epoxydienes (Millar et al., 1987). The purity of the produced (Z,Z)-3,9-*cis*-6,7-epoxynonadecadiene was 98% with respect to the other positional isomers.

Electrophysiology. Electrophysiological recordings were made on excised male antennae. An antenna was placed with the base in a pipet electrode filled with Beadle-Ephrussi Ringer, and grounded via an Ag–AgCl wire. For electroantennographic recordings (EAG), the distal tip of the antenna was placed in contact with a second electrode, similar to the indifferent electrode. The tip electrode was connected to a high-impedance DC amplifier with automatic baseline drift compensation.

For single sensillum measurements, an olfactory sensillum was cut by means of two glass knives, and a recording electrode was placed in contact with the cut surface of the sensillum. The recording electrode was a glass pipet with a tip diameter of about 4 μm, filled with Ringer, thickened Ringer (PVP), and finally coated with a layer of Vaseline to prevent evaporation. The recording electrode was connected to a high-impedance AC amplifier (Hansson et al., 1987). In stationary recordings, 10 μg of a synthetic compound was applied to filter paper with a disposable 5-ml plastic syringe. Two milliliters of the syringe atmosphere was injected into a purified and moistened airstream flowing over the antenna at a speed of about 0.5 m/sec.

Field Tests. Preliminary field tests were conducted using sticky traps (Arn et al., 1979) and established methods (Tóth et al., 1987).

RESULTS

Chemical Analysis. The extract from *E. defoliaria* females was first investigated by simultaneous FID-EAD. Gas chromatographic separation showed two compounds that elicted clear responses from the male antenna (Figure 1). Two FID-SC analyses showed an inhibition of the spontaneous activity by the first EAD active component, and a strong stimulation by the second EAD active component (Figure 2). On a DB1 column, the equivalent acetate chain lengths of the first and second active components were 1477 and 1639, respectively. When compared to available synthetic substances, the retention time of the first component coincided exactly with that of *Z3,Z6,Z9*–19Hy, and the second component matched the retention time of (Z,Z)-6,9-nonadecadien-3-one, a sex pheromone component of *Peribatodes rhomboidaria* Den. & Schiff. (Buser et al., 1985). When analyzed on a DB wax column, component I again matched the retention time of *Z3,Z6,Z9*–19Hy. However, the second component did not coincide with the retention time of the ketone on this column.

Combined GC-MS analyses of the two EAD active peaks showed that the mass spectrum of the first component ($M^+ = 262$) exactly matched that of

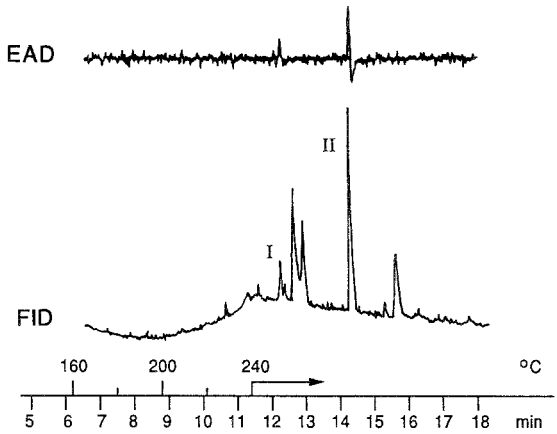

FIG. 1 GC-FID chromatogram from analysis of a female *Erranis defoliaria* pheromone gland extract and corresponding EAD response from an antenna of a conspecific male. Two peaks (I and II) show EAD activity.

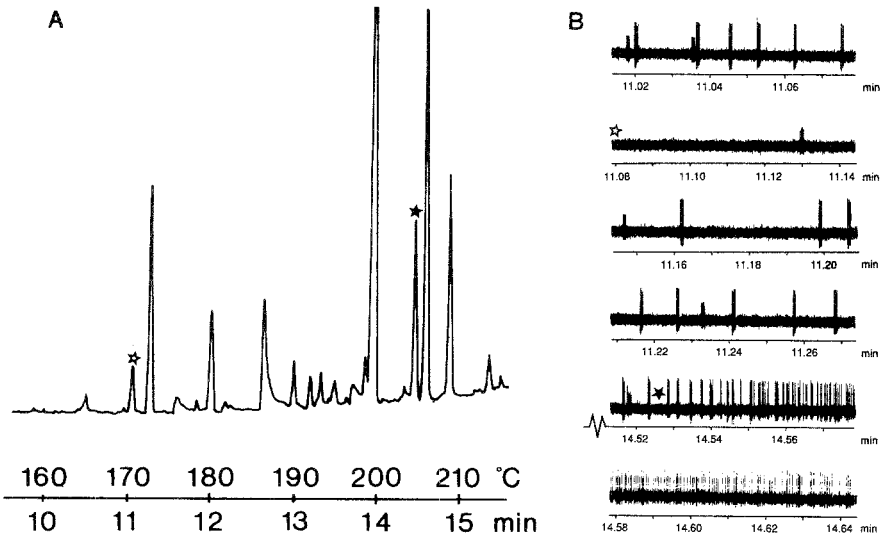

FIG. 2. GC-FID chromatogram from analysis of a female pheromone gland extract (A), and simultaneously recorded single sensillum response from a male olfactory sensillum in *Erannis defoliaria* (B). The retention time of *Z*3,*Z*6,*Z*9–19Hy and the simultaneous onset of the stimulation of the single sensillum preparation is shown by a white star. The retention time of and stimulation by *Z*3,*Z*9-*cis*-6,7-epo-19Hy is shown by a black star.

*Z*3,*Z*6,*Z*9–19Hy (Becker et al., 1983). The mass spectrum of the second compound (M^+ = 278; $C_{19}H_{34}O$ according to high-resolution GC-MS) showed a base peak at m/z = 67 and diagnostic fragmentation with relatively intense signals in the lower mass region (Figure 3). Assuming close biogenetic relations between the two active compounds, the structure of the second component was more likely to be that of an epoxydiene than that of a ketodiene, since no characteristic acylium ion or product of a McLafferty rearrangement could be detected.

Of the three monoepoxydienes derived from *Z*3,*Z*6,*Z*9–19Hy, (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene and (*Z*,*Z*)-3,6-*cis*-9,10-epoxynonadecadiene showed very different mass spectra when compared to the natural compound (Figure 3). However, the mass spectrum and gas chromatographic retention times (on three different columns) of synthetic (*Z*,*Z*)-3,9-*cis*-6,7-epoxynonadecadiene exactly matched the natural product. The enantiomeric configuration of the active pheromone component remains to be established. In each female about 1.5 ng of the main component (*Z*,*Z*)-3,9-*cis*-6,7-epoxynonadecadiene was found.

Electrophysiology. The activity of the synthetic pheromone components was tested by both EAG and single sensillum experiments. FID-EAD runs with 5 ng of the synthetic substances showed a strong EAG response to both of them. The component elicting the strongest response was the *Z*3,*Z*9-*cis*-6,7-epo-19Hy.

The single sensillum measurements, made on 12 individual sensilla, revealed two physiological types of sensilla. In type I, a large spike amplitude cell responded to the epoxide and a small spike amplitude cell responded to the triene. In the type II sensillum, only one cell responded. It responded to the epoxide with a large spike amplitude cell, but was inhibited by the triene component (Figures 2 and 4). A small cell could be seen also in the type II receptor, but it was not stimulated by any of the pheromone components.

Field Tests. Preliminary field trapping experiments performed in Hungary resulted in substantial trap catches of male mottled umber moths with a mixture of the epoxide and the triene (Table 1).

In the course of the field tests, males of another geometrid, *Colotois pennaria* L., were caught with the same bait (Table 1). Supplementary FID-EAD and GC-MS analyses proved *Z*3,*Z*9-*cis*-6,7-epo-19Hy to be present in female pheromone gland extracts of *C. pennaria* as well. This species also is regarded as a member of the pest complex of autumn-flying geometrids (Balas and Saringer, 1982). More detailed results concerning the identification of the pheromone of this species will be published elsewhere.

In another field test, conducted in the early spring of 1989, in Hungary, high numbers of a spring-flying geometrid, *Agriopis marginaria* F, a close relative of *E. defoliaria*, were captured (Table 1). *A. marginaria* is regarded as a potential orchard pest in Central Europe (Balas and Saringer, 1982).

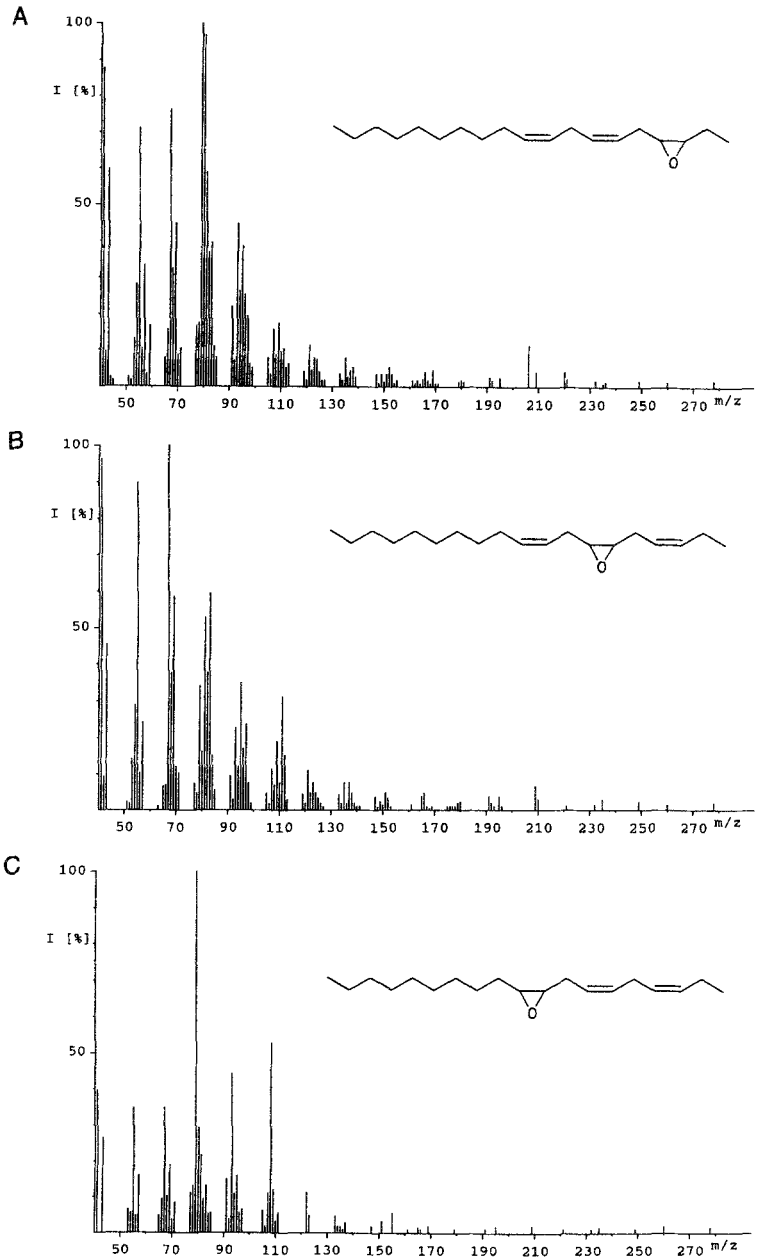

FIG. 3. Electron impact mass spectra (70 eV) of (*Z*,*Z*)-6,9-*cis*-3,4-epoxy-nonadecadiene (A), (*Z*,*Z*)-3,9-*cis*-6,7-epoxy-nonadecadiene (B), and (*Z*,*Z*)-3,6-*cis*-9,10-epoxy-nonadiene (C).

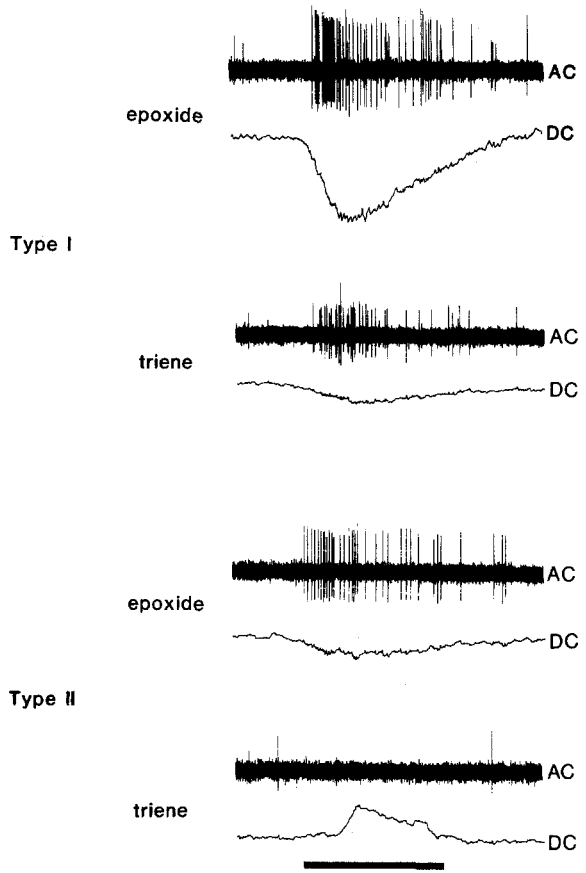

FIG. 4. Single sensillum responses from two physiologically different types (I and II) of male pheromone sensitive sensilla to the two identified pheromone components Z3,Z6,Z9–19Hy and Z3,Z9-*cis*-6,7-epo-19Hy. The horizontal black bar indicates the stimulation time (1 sec).

TABLE 1. FIELD CATCHES OF MALE GEOMETRIDS IN TRAPS BAITED WITH MIXTURE OF Z3,Z9-*cis*-6,7-epo-19Hy AND Z3,Z6,Z9–19Hy IN PRELIMINARY TESTS IN HUNGARY[a]

Z3,Z6,Z9–19Hy (μg)	Z3,Z9-*cis*-6,7-epo-19Hy (μg)	Total number of males caught		
		Erannis defoliaria	*Agriopis marginaria*	*Colotois pennaria*
0	0	1	0	0
30	100	123	98	54

[a]Tests conducted from November 1, 1988 to March 13, 1989, near Budapest in six replicates.

DISCUSSION

With regard to pheromone composition, the only well-investigated geometrid occurring in Europe is *Operophtera brumata* L., the winter moth. Independently, Roelofs et al. (1982) and Bestmann et al. (1982) reported a pheromone consisting of a single component (*Z*,*Z*,*Z*)-1,3,6,9-nonadecatetraene. While in *O. brumata*, *Alsophila pometaria* Harr. (Wong et al., 1984), and *Sabulodes caberata* Guenée (McDonough et al., 1986) the pheromone seems to consist of hydrocarbons only, other species produce mixtures of hydrocarbons and their corresponding oxygen-containing derivatives. The sex pheromone of *P. rhomboidaria* is a mixture of *Z*3,*Z*6,*Z*9–19Hy and (*Z*,*Z*)-6,9-nonadecadien-3-one (Buser et al., 1985), while *Boarmia selenaria* Schiff. produces (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene instead of the ketone (Becker et al., 1987). In the present study the pheromone of *E. defoliaria* showed the same dual principle as it consisted of the nonadecatriene found in the former species, along with a new epoxide, *Z*3,*Z*9-*cis*-6,7-epo-19Hy, as the oxygen-containing component.

The single sensillum recordings reported in this paper are the first published on geometrid male responses to the female sex pheromone. One of the sensillum types encountered (type I) showed a type of response that is often encountered in various moth orders (Den Otter, 1977; Hansen, 1984; Priesner et al., 1984; Hansson et al., 1987): a large spike amplitude cell responding to the main pheromone component in the female pheromone gland extract and a small spike amplitude cell responding to the minor component. However, a second type of sensillum was discovered. This type of sensillum (type II) responded with a large spike amplitude to the main component, but was inhibited by the minor component. As far as we know, this phenomenon has not been observed in the relationship between pheromone components at the peripheral reception level. It has been shown that plant substances can inhibit olfactory cells, as in the interaction between (*Z*)-11-tetradecenyl acetate and geraniol in male *Yponomeuta evonymellus* L. (Yponomeutidae) pheromone receptors (Van Der Pers et al., 1980), but the specific action of one pheromone component inhibiting the response to another is new. This gives the moth a second source of information about the exact proportions of the pheromone components. The type II cell could enable the male to assess the concentrations of the components in the pheromone blend emitted by the female very exactly. Too much epoxide would give a response that is too strong, and too much triene would decrease the response. A delicate balance between the two components would be needed to elicit the "right" frequency of action potentials in the olfactory cell.

It is interesting to note that *Z*3,*Z*9-*cis*-6,7-epo-19Hy, in common with many other components of moth pheromones, is not a species-specific compound. We also identified it as a sex pheromone component of *C. pennaria* and

found it to be attractive to *A. marginaria*, which indicates that this compound may be a generally occurring pheromone component among geometrids. Field tests have shown that males of other geometrid species also were attracted to this type of compound. Wong et al. (1985) showed in an extensive field-trapping and electroantennographic study that males of 17 geometrids were caught in traps baited with different mixtures of triene hydrocarbons and diene epoxides. However, the enantiomeric configuration has not been established and might be an important factor in the species specificity of the sex pheromones in these species.

To our knowledge, *Z*3,*Z*9-*cis*-6,7-epo-19Hy has not been found in the sex pheromone of any other species; neither has there been any published demonstration of its field activity towards male Lepidoptera. However, its shorter homolog, (*Z*,*Z*)-3,9-*cis*-6,7-epoxy-heptadecadiene has been reported to catch male *Semiothisa delectata* Hulst. in mixtures with the parent hydrocarbon (*Z*,*Z*,*Z*)-3,6,9-heptadecatriene (Millar et al., 1987), and in *S. ulsterata* Pearsall its occurrence in the female pheromone gland extract has been demonstrated by comparison of retention times and electrophysiological activity of peaks (Millar et al., 1987.

We conclude that the mixture of *Z*3,*Z*6,*Z*9-19Hy and *Z*3,*Z*9-*cis*-6,7-epo-19Hy offers a useful basis for optimization of a practically applicable attractant to catch males of *E. defoliaria*. Further studies of the species specificity of attractant mixtures and determination of the absolute configuration of the natural epoxide are in progress.

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HONEYDEW ANALYSIS FOR DETECTING PHLOEM TRANSPORT OF PLANT NATURAL PRODUCTS
Implications for Host-Plant Resistance to Sap-Sucking Insects

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Abstract—Analysis of honeydew excreted by various sap-sucking insects indicated the presence of certain plant secondary constituents in the phloem of their host plants. Honeydew excreted by mealybugs (*Pseudococcus longispinus*), living on *Castanospermum australe*, contained the indolizidine alkaloid castanospermine, a potent β-glucosidase inhibitor. Similarly, honeydew from green peach aphids (*Myzus persicae*), feeding on *Senecio vulgaris* flower buds, contained the pyrrolizidine alkaloid senecionine, its *N*-oxide, and hydrolytic products including retronecine. Cardenolides were detected in the honeydew of oleander aphids (*Aphis nerii*) feeding on oleander (*Nerium oleander*), indicating that these compounds are translocated in the phloem. On the other hand, honeydew from greenbugs (*Schizaphis graminum*), feeding on barley, lacked gramine or related indole metabolites. Similarly MBOA, the breakdown product of DIMBOA, was not detected in the honeydew of greenbugs living on DIMBOA-containing wheat.

Key Words—*Castanospermum australe*, Moreton Bay chestnut, *Senecio vulgaris*, groundsel, *Pseudococcus longispinus*, mealybug, aphid, *Schizaphis graminum*, greenbug, *Myzus persicae*, green peach aphid, *Aphis nerii*, oleander aphid, Homoptera, Aphididae, Coccidae, phloem transport, honeydew, indolizidine, pyrrolizidine alkaloid, gramine, DIMBOA, cardenolide.

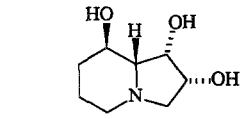
INTRODUCTION

Although an immense number of secondary natural products encompassing numerous structural types are known, much less is known about their sites of biosynthesis, transport, turnover, and storage in plants. The available methods for studying transport and storage of natural products are normally limited to cytochemical techniques, analysis of isolated cell compartments and tracer labeling procedures (Waller and Nowacki, 1978; Lucker et al., 1980).

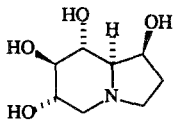
The site of natural product biosynthesis and accumulation varies with plant species and class of compound. In cases where the sites of production and storage of these materials are not the same, translocation must be involved. Material transport in plants occurs in the vascular bundle. The phloem transports primarily sucrose, and to a lesser degree amino acids, from the site of biosynthesis in the leaves to other plant parts. The xylem transports primarily water and minerals from the roots to the aerial parts of the plant.

Excretion of honeydew by sap-feeding insects corresponds with ingestion of phloem sap (Mittler, 1958). Hence, in lieu of direct analysis of phloem sap, honeydew can provide evidence for natural product transport in the phloem. A previous paper from these laboratories reported (Dreyer et al., 1985) the occurrence of the indolizidine alkaloid swainsonine (**1**) (Figure 1) in the honeydew (excreta) of pea aphids [*Acyrthosiphon pisum* (Harris)] feeding on swainsonine-producing *Astragalus* (Leguminosae) species (Molyneux et al., 1986). Since the pea aphid is a known phloem feeder, these results establish that swainsonine must be biosynthesized in the leaves and transported in the phloem. This information now has been utilized to establish the most efficient method of feeding radiolabeled pipecolic acid to *A. oxyphysus* in order to determine the biosynthesis of swainsonine in the plant (Harris et al., 1988). Similar results have been obtained by Wink and co-workers (1982, 1985, 1986) with aphids feeding on various quinolizidine alkaloid producing legumes. Campbell and Binder (1984) reported the occurrence of pinitol in the honeydew of pea aphids feeding on alfalfa, while Kiss (1984) found evidence for quaternary furanoquinoline alkaloids in the honeydew of leafhoppers [*Vanduzeea arquata* (Say) and *Echenopa binotata* (Say)] feeding on *Ptelea* (Rutaceae). Glucosinolate levels in the honeydew of cabbage [*Brevicoryne brassicae* (L.)] and peach aphids [*Myzus persicae* (Sulzer)] feeding on different rape [*Brassica napus* (L.)] cultivars parallel the levels in the plant (Weber et al., 1986). Examination of aphid gut contents has also been employed to study phloem transport of exogenous phenolics (MacLeod and Pridham, 1965; see also Bornman and Botha, 1973; Eschrich, 1966, 1968).

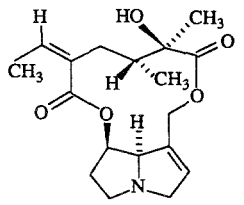
These observations have now been extended to several related systems, which demonstrate that, in selected cases, the analysis of insect honeydew can yield useful insights into the phloem transport of natural products and, by infer-



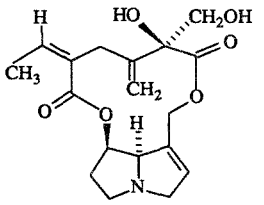
SWAINSONINE (1)



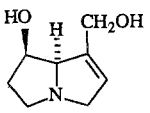
CASTANOSPERMINE (2)



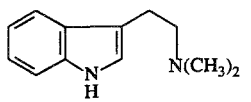
SENECIONINE (3)



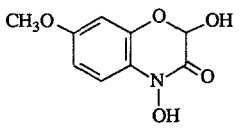
RIDDELLIINE (4)



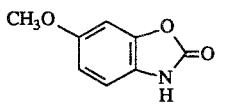
RETRONECINE (5)



GRAMINE (6)



DIMBOA (7)



MBOA (8)

FIG. 1. Structures of natural products investigated for potential transport in phloem.

ence, their site of biosynthesis (see also: Peel, 1975; Dixon, 1975). It is fortunate that a sap-sucking insect of some sort can be found as a probe for a surprisingly large number of plants. The appropriate controls for the possible metabolic transformation of the natural product in the insect can be obtained by adding the substance to an artificial diet and analyzing the honeydew of aphids feeding thereon. An alternate, but far more difficult approach, is to examine exudates collected from stylets severed while the tips are located in the phloem.

Finally, it may be possible in the course of such experiments to acquire some knowledge about the biological relevance of secondary natural products in the phloem by observations of the behavior of the insect on the plant, e.g.,

feeding deterrency, reproduction rates, sequestration, metabolic transformations, and structural requirements for phloem transport. Conclusions about the possible ecological roles of such compounds may thus be drawn.

The transport of natural products via the phloem could potentially play a major role in the ecology of and host plant resistance towards sap sucking insects. Although a great many substances have been tested as aphid feeding deterrents by addition to artificial diets (Dreyer and Jones, 1981, and references cited therein), their occurrence in the pholem is unlikely in most cases (Matile, 1984). Even though some of these substances are highly active feeding deterrents, this great diversity of compounds may not play a significant ecological role against sap-sucking insects unless they occur in the phloem. This is because the quasiintercellular mode of aphid probing enables them to avoid these compartmentalized materials.

It is the purpose of this paper to demonstrate the utility of analyzing homopteran honeydew as a technique, whenever a favorable situation occurs, to study the occurrence of secondary metabolites in plant vascular tissues.

METHODS AND MATERIALS

Honeydew Collection. Honeydew was collected by placing sheets of aluminum foil around the base of barley plants colonized by greenbugs, biotype E. After several days, when a sufficient amount of honeydew had been deposited, the foil was cut up into a beaker and washed with hot 20% aqueous methanol. Solvent was removed from the methanol extracts and the residue taken up in ethyl acetate. The ethyl acetate extracts were partitioned with 5% HCl. The HCl extracts were made basic with dil. NaOH and extracted with methylene chloride. After drying, the extracts were concentrated. This concentrate was used for spotting on 0.25-mm silica gel 60 thin-layer plates using an EtOAc—i-PrOH—NH_4OH (45 : 35 : 20) solvent system. The extract was run against a reference sample of gramine, as well as the alkaloid fraction from similarly prepared barley extracts. Compounds of interest were detected by spraying with Ehrlich's reagent, followed by fuming with HCl gas, to give blue-pink colors characteristic of simple indoles.

Honeydew of mealybugs on small, 1 to 2-year-old, Moreton Bay chestnut trees was collected for several days on aluminum foil placed under the leaves. The foil was washed with hot aq. methanol and the extracts chromatographed on 0.25-mm silica gel 60 thin-layer plates, using a solvent system of $CHCl_3$—MeOH—NH_4OH—H_2O (35 : 13 : 1 : 1), with authentic castanospermine as a standard. The TLC plate was sprayed with 10% acetic anhydride in benzene, warmed on a steam bath until the odor of acetic anhydride was absent, and then sprayed with Ehrlich's reagent (2 g *p*-dimethylaminobenzaldehyde in

100 ml of EtOH containing 2 ml BF_3 etherate) followed by heating on a hot plate until fuming ceased. Characteristic purple spots are formed by indolizidine alkaloids which are capable of forming pyrroles by dehydration, such as swainsonine and castanospermine (Molyneux et al., 1986).

In a similar fashion, honeydew of green peach aphids on *S. vulgaris* was collected and analyzed for pyrrolizidine alkaloids by TLC using a specific spray reagent (Molyneux and Roitman, 1980).

Honeydew from greenbugs on wheat was collected as described above for barley. The ethanol-soluble honeydew fraction was chromatographed against a reference sample of 6-methoxy-1,4-benzoxazinolone (MBOA) (**8**) on 0.25-mm silica gel 60 thin-layer plates in EtOAc—$CHCl_3$ (1 : 1). There was no evidence of MBOA in the honeydew.

Honeydew from oleander aphids [*A. nerii* (Fonscolombe)] on oleander plants was collected on aluminum foil and analyzed by silica gel TLC in EtOAc—MeOH (97 : 3) using 2,2′,4,4′-tetranitrodiphenyl as the visualization reagent (Brower et al., 1982).

Feeding Deterrency of Castanospermine. The diet and bioassay procedures used to measure feeding deterrency have been described previously (Dreyer et al., 1981). The mealybugs gave relatively large amounts of honeydew on the artificial diet.

RESULTS AND DISCUSSION

Phloem Transport of Castanospermine. Castanospermine (**2**) is an indolizidine alkaloid closely related in structure to swainsonine, the primary toxin of locoweeds. It is a potent α- and β-glucosidase inhibitor (Saul et al., 1984, 1985) and is an intense feeding deterrent (ED_{50} = 20 ppm) for the pea aphid when incorporated into an artificial diet (Dreyer et al., 1985). A number of attempts to colonize the castanospermine-containing Moreton Bay chestnut, *Castanospermum australe* (Leguminosae), with the pea aphid failed. However, a small tree of Moreton Bay chestnut growing in the laboratory was opportunistically colonized by the long-tailed mealybug, *Pseudococcus longispinus* (Targioni-Tozzetti). When honeydew was collected from these mealybugs feeding on Moreton Bay chestnut and analyzed by TLC, clear evidence for the presence of castanospermine was obtained.

In order to develop the necessary controls, it was established that the mealybug would feed at modest levels through Parafilm on the Akey diet (Akey and Beck, 1971), an artificial diet normally used for aphids (Dreyer et al., 1981). The number of mealybugs feeding on the control diets (ca. 60%) was not as high as that of aphids ($\geq$90%), but this difference could be a result of the chamber configuration as well as diet composition. When castanospermine was

added to this artificial diet at levels up to 100 ppm, there was no decrease in the number of mealybugs feeding relative to the number feeding on controls lacking castanospermine. The feeding by mealybugs on plants and diets that contain castanospermine is intriguing in view of the fact that castanospermine is a potent inhibitor of a number of β-glucosidases, including sucrase, found in this mealybug (Campbell et al., 1987). Thus, castanospermine, a potential metabolic toxin, is not a feeding deterrent to the mealybug, in contrast to the situation with the pea aphid. The large amounts of honeydew produced and the alignment of the mealybugs along the leaf veins on the plant indicate that the long-tailed mealybug is a phloem feeder. These results support the view that, like swainsonine, castanospermine is biosynthesized in the leaves and is transported in the phloem of the Moreton Bay chestnut.

Phloem Transport of Pyrrolizidine Alkaloids. During efforts to expand the scope of this approach in determining phloem occurrence of secondary natural products, it proved possible to get green peach aphids to colonize *Senecio vulgaris* (L.) (Compositae), a pyrrolizidine alkaloid-containing weed species. Pyrrolizidine alkaloids could be detected readily in the plant. The plant contained primarily senecionine (**3**) and its *N*-oxide, but the more polar alkaloids, such as riddelliine (**4**), were not detectable in the plant material used. TLC (Molyneux and Roitman, 1980) of the honeydew showed two spots reacting positively with Ehrlich's reagent, one having an R_f similar to but not identical with riddelliine, and the second corresponding to retronecine (**5**), the necine base moiety of the macrocyclic pyrrolizidine alkaloids in *S. vulgaris*. In addition, senecionine *N*-oxide was present. The presence of retronecine in the honeydew requires that the aphid possess a gut esterase system (MacLeod and Pridham, 1965) that hydrolyzes the macrocyclic diester ring. Since two ester groups must be hydrolyzed to produce retronecine, the unknown substance in the honeydew is possibly a result of hydrolysis of only one of the ester groups. The complete structural elucidation of this metabolite must await the isolation of sufficient material from honeydew samples. Apparently, similar ester hydrolysis of senecionine *N*-oxide does not occur.

Hartmann and Toppel (1987) have demonstrated that root cultures of *S. vulgaris* biosynthesize senecionine *N*-oxide, which does not undergo turnover and accumulates as a stable metabolic product. The water solubility of the *N*-oxide relative to the free base makes it admirably suited for phloem transport and thereby mobilized for whatever ecological function it may have.

Lack of Phloem Transport of Gramine in Barley. Corcuera (1984) has proposed that the presence of gramine (**6**) is a possible factor in host-plant resistance of barley (Gramineae) towards the greenbug, *Schizaphis graminum* (Rondani), an aphid that can be an important pest on barley. Gramine shows modest feeding deterrency towards the greenbug when added to an artificial diet (Zuniga et al., 1985). It has been established that the greenbug is a phloem

feeder and probes intercellularly (Campbell et al., 1982). The ability to probe intercellularly enables aphids to avoid compartmentalized secondary natural products in plants that might otherwise have a deleterious effect on the insect and consequently play a role in host-plant resistance. Thus, for gramine to exercise an influence in host-plant resistance towards aphids, it must occur either in the middle lamella or in the phloem.

TLC, using Ehrlich's reagent for detection, as well as GC-MS analysis, of honeydew from greenbugs (biotype E) feeding on barley failed to disclose the presence of gramine. With the same analytical methods, gramine and *N*-methyltryptamine were clearly present in the plant extracts. Greenbugs feeding on an artificial diet containing gramine at the ED_{50} level (500 ppm) failed to produce honeydew, so desirable controls were not obtained. However, TLC analysis of the extracts of whole aphids after feeding on barley failed to demonstrate the presence of gramine, showing that it is not sequestered intact by the insect. Gramine perhaps exerts its effect on greenbugs in an as yet unknown manner. Nevertheless, a feeding-deterrent role for gramine in host-plant resistance of barley is questionable since the compound appears to be compartmentalized and avoided by the aphids.

Hanson et al. (1983) have argued that gramine will tend to migrate to and accumulate in compartments with low pH and that gramine-accumulating barley lines may suffer autotoxic effects. The failure to find gramine in greenbug honeydew may be explained by the possibility that it is translocated in the xylem and not the phloem. The site of gramine biosynthesis is at the base of the seedling leaf from where it is subsequently translocated to the leaf tip (Smith, 1977; Schuette, 1972). Since this translocation is upwards, gramine most probably occurs in the xylem, which is consistent with its absence in the aphid honeydew. However, Argandoña et al. (1987) were unable to detect gramine in the vascular bundles separated from barley leaf tissues. In a similar manner, Montgomery and Arn (1974) failed to find the dihydrochalcones, phlorizin and phloretin, in honeydew of the apple aphid, *Aphis pomi*, or the insect itself feeding on phlorizin-containing apple trees. They concluded that phlorizin was not present in the phloem.

Lack of Phloem Transport of DIMBOA Glucoside in Wheat. Argandoña et al. (1980, 1981, 1982, 1983; Argandoña and Corcuera, 1985) have maintained that the hydroxamic acid, DIMBOA (**7**), plays a significant role in host-plant resistance towards greenbugs on wheat . DIMBOA (2,4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one) is known to be a feeding deterrent towards aphids when added to an artificial diet (Argandoña et al., 1983), and hydroxamic acids have been shown to be present in the vascular bundles of wheat leaves (Argandoña et al., 1987). However, a role for DIMBOA in host-plant resistance in all wheat varieties is debatable. First, it is well established that DIMBOA levels are high only in seedling wheat and that the concentration drops rapidly as the plant

matures. Thus, DIMBOA cannot account for resistance in greenbug-resistant wheat lines that maintain their resistance well into maturity, a stage when DIMBOA levels are extremely low or even undetectable. Second, since DIMBOA occurs as a glucoside, it must be compartmentalized in order to prevent its hydrolysis by endogenous β-glucosidase (Matile, 1984). Moreover, DIMBOA is not stable in solution and readily breaks down into MBOA (6-methoxybenzoxazinolone) (**8**). This decomposition to MBOA is more rapid in the presence of a β-glucosidase. The presence of β-glucosidases in green bugs is well established (Campbell et al., 1987), and MBOA is not a feeding deterrent to greenbugs. Our failure to detect MBOA in the honeydew supports these arguments against DIMBOA as the host-plant resistance factor to greenbugs feeding on wheat.

Cardenolides in Honeydew of Oleander Aphid. The oleander aphid has been shown to probe intercellularly and to be a phloem feeder on species of Asclepiadaceae (Botha et al., 1972). The large amount of honeydew produced by this aphid when feeding on oleander [*Nerium oleander* (L.)] indicates that it is also a phloem feeder on this host. Previous work (Rothschild et al., 1970) on *A. nerii* feeding on cardenolide-containing oleander showed that this aphid species had the ability to sequester cardenolides. However, the pattern of cardenolides found in the aphid did not match those found in the plant. Apparently the insects have the ability to modify the cardenolide system, presumably by the removal or addition of sugars to the aglycone and/or the cardenolides transported in the phloem. A preliminary inspection of honeydew of oleander aphids feeding on host oleander, using TLC with a selective detection reagent for 2-butenolide-containing cardenolides (Brower et al., 1982), showed their presence in the honeydew. However, the pattern of spots was different from those found in the host-plant extracts, and the composition of the cardenolides in the honeydew requires further investigation. It is noteworthy that the cardenolide content of the aphids does not protect them from ladybugs (Rothschild et al., 1970) nor does it deter ants from tending the olender aphids for their honeydew (Jones, unpublished observations).

The examples cited above are presented in order to illustrate the potential of honeydew analysis for the study of phloem transport of natural products. In those examples where a natural product identified in the plant is also detected in the honeydew, the conclusion is unequivocal, namely, that the metabolite must be transported in the phloem, provided that the possibility of biosynthesis by the insect itself can be excluded. In those cases where the result is negative, the situation must be carefully scrutinized. In our study, we suggest that such constituents may be compartmentalized (e.g., DIMBOA) or translocated in the xylem (e.g., gramine). It is obvious that natural products that are not appreciably water soluble are unlikely to be transported in the phloem unless they are conjugated as glycosides, sulfates, etc. However, it is possible that phloem-

transported metabolites may be degraded or transformed by the aphid to such an extent that they are not detected in the honeydew by the analytical method chosen. Alternatively, the compound may be sequestered rather than secreted in the honeydew. The possibility of sequestration can be investigated by crushing the aphids and analyzing the extracts of the homogenate.

Since many plants of interest to phytochemists may become fortuitously colonized by sap-sucking insects to which they are not normally host, advantage should be taken of this phenomenon. The techniques of honeydew collection and analysis thereof, exemplified by the above examples, are relatively uncomplicated. Valuable information as to the transport or compartmentalization of secondary plant metabolites may be obtained by untilization of these methods. It should be noted that the potential exists for application of similar techniques to the study of translocation of systemic herbicides.

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ASARONES FROM *Acorus calamus* L. OIL
Their Effect on Feeding Behavior and Dietary Utilization in *Peridroma saucia*

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Abstract—Asarones (2, 4, 5-trimethoxypropenylbenzenes) isolated from the essential oil of *Acorus calamus* L. rhizomes, are potent growth inhibitors and antifeedants to the variegated cutworm, *Peridroma saucia* Hubner. *cis*-Asarone added to artificial diet significantly inhibited growth and feeding by first-, third-, and fourth-instar larvae, whereas the *trans* isomer produced an antifeedant effect alone. Gross dietary utilization (efficiency of conversion of ingested food, ECI) was decreased when the diet was supplemented with *cis*-asarone or when this compound was topically applied to fourth-instar larvae. Inhibition of growth occurred even at a moderate topical dose (5 μg/larva) primarily as a result of decreased efficiency of conversion of digested food (ECD), even though the approximate digestibility (AD) of the food was unchanged. Oral or topical treatment with *trans*-asarone also significantly inhibited larval growth, but in this case the effect can be strictly attributed to decreased consumption, as dietary utilization (ECI) was not affected. Both isomers displayed a direct antifeedant effect based on leaf disk choice tests. The *cis* isomer was 7.0 and 5.5 times more potent thant the *trans* isomer against fourth- and fifth-instar larvae, respectively. Our data suggest that the two asarone isomers have different modes of action. *cis*-Asarone is toxic in addition to having strong antifeedant activity, whereas the *trans* isomer acts only as an antifeedant with no appreciable toxicity.

Key Words—*Peridroma saucia*, variegated cutworm, Lepidoptera, Noctuidae, feeding behavior, growth inhibitors, conversion efficiency, asarones, *Acorus calamus*, antifeedants.

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INTRODUCTION

The essential oil from the rhizomes of the sweet flag, *Acorus calamus* L. (Araceae), has been shown to exhibit residual and contact toxicity against several insect species of economic importance (Dixit et al., 1956; Paul et al., 1965; Deshmukh and Borle, 1975; Pandey et al., 1977; Teotia and Tewari, 1977; Teotia and Pandey, 1979; Rajendran and Gopalan, 1979). The antigonadal activity of this oil against both sexes of various thysanuran, coleopteran, heteropteran, dipteran, and hymenopteran insects also has been documented (Saxena and Rhodendorf, 1974; Mathur and Saxena, 1975; Koul et al., 1977a,b; Saxena et al., 1977; Koul, 1979; Matolcsy et al., 1981; Schmidt and Brochers, 1981; Ramos-Ocampo and Hsia, 1986). Antigonadal activity of the oil is attributable to the phenylpropene *cis*-asarone (Saxena et al., 1977; Koul, 1979; Matolcsy et al., 1981; Schmidt and Brochers, 1981; Ramos-Ocampo and Hsia, 1986), which causes inhibition of egg production and other gonadal dysfunctions through suppression of interstitial cell and trophocyte secretions (Koul, 1979).

A. calamus oil also inhibits growth and feeding in two species of polyphagous lepidoptera, *Spodoptera litura* F. and *Peridroma saucia* Hubner (Koul, 1987; Koul and Isman, 1990). This paper reports studies identifying the active oil constituents responsible for these bioactivities and examines the behavioral and physiological responses of *P. saucia* to these active compounds.

METHODS AND MATERIALS

A. calamus oil (bp 163–165°C, $n_D^{25} = 1.5535$) was distilled from rhizomes as previously described (Koul, 1987). Gas chromatography revealed the presence of a major peak (ca. 72% of the total oil) along with several minor components. Fractional distillation of the oil allowed isolation of the major peak to >98% purity, and this fraction was found to be responsible for the bioactivity of the oil. This active fraction was identified as a mixture of *cis*- and *trans*-asarones (Figure 1) using IR and NMR spectroscopies as described by Rao and Subramaniam (1937). Separation of the *cis* and *trans* isomers of asarone was achieved using a Waters (model 840) high-performance liquid chromatograph equipped with a reverse-phase column (15 cm × 3.9 mm ID, Nova-Pak C_{18}, Waters Associates) using UV detection at 254 nm. A linear gradient from 30% aqueous acetonitrile to 100% acetonitrile over 40 min was run at a flow rate of 0.6 ml/min. Both isomers thus separated by HPLC (Figure 1) were spiked with commercial samples (Sigma Chemical Co., St. Louis, Missouri) that chromatographed exactly with the peaks separated from the candidate oil.

Variegated cutworm larvae from a laboratory colony were used for the study. All insects were maintained at 27 ± 1°C and 16:8 light–dark and reared

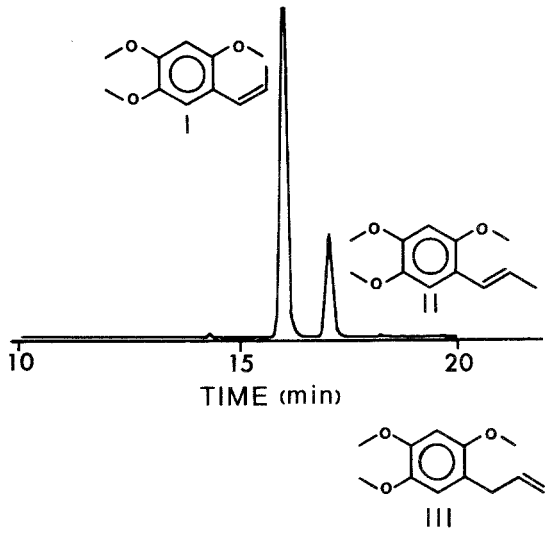

FIG. 1. High-performance liquid chromatogram of active fraction from *Acorus calamus* oil showing peaks for *cis*-asarone (I) and *trans*-asarone (II). Isoasarone (III) is shown for comparison.

on an artificial diet purchased from Bioserv Inc. (Frenchtown, New Jersey) until transferred to experimental diets.

Experimental diets contained *cis*- or *trans*-asarone at concentrations of 60–2000 ppm fresh weight (fwt) of diet. Bioassays were initially performed with first- (neonate) and third-instar larvae (20 larvae per concentration) as previously described (Koul and Isman, 1990). Larval weights and mortality were recorded after seven and 10 days for first-instar larvae and after two, four, six, and eight days for third-instar larvae.

Nutritional experiments were carried out with fourth-instar larvae as these larger larvae and their frass could be more accurately weighed than that of the first three instars. In the first experiment, 20 larvae/concentration were provided with either compound at dietary concentrations of 250–1000 ppm as previously described (Koul and Isman, 1990). Relative growth per unit weight of the insect at the outset of the experiment (RGRi) and relative consumption per unit weight of the insect at the outset of the experiment (RCRi) were calculated on a dry weight basis (Farrar et al., 1989) after three days of feeding. Indices of food conversion efficiency used are defined as follows: (1) approximate digestibility (AD) % = 100 $(C - F)/C$; (2) efficiency of conversion of ingested food (ECI) % = 100 × G/C; (3) efficiency of conversion of digested food (ECD) % = 100 × $G/(C - F)$; where C = dry weight of food consumed, F = dry weight of frass produced, and G = dry weight gain of insect.

cis- and *trans*-Asarone were also topically applied to fourth-instar larvae in 1 μl acetone at doses of 5, 10, 20, and 30 μg/larva, with appropriate controls treated with acetone alone. Twenty larvae were treated with each compound at each dose and were then allowed to feed on untreated diet for 72 hr before insects, remaining food, and frass were dried at 60°C to constant weight, and nutritional indices calculated.

Antifeedant activity was assayed using a leaf disk choice test (Koul and Isman, 1990). The 2.0-cm^2 (mean fresh weight, 62.0 mg) disks were punched out from cabbage leaves (*Brassica oleracea* var. Stonehead) and treated on each side with 15 μl of aqueous asarone solutions emulsified with Triton X-100 (0.1%) to give concentrations ranging from 1.0 to 45.0 μg/cm^2. Controls were treated with 15 μl of the carrier alone. The leaf disks were dried at room temperature and then fourth- or fifth-instar larvae were introduced into each arena (9 cm diameter) containing two treated and two control disks in alternate positions. Experiments were carried out with two larvae per Petri dish with five replicates for each treatment. Consumption was recorded using a digitizing leaf area meter (model LI-3000, Li-Cor,) after 20 hr for fourth-instar and 6 hr for fifth-instar larvae. The index of feeding deterrency for each treatment was calculated as $(C - T)/(C + T) \times 100$, where C is the consumption of control disks and T the consumption of treated disks.

Data were analyzed using analysis of variance, linear regression, and probit analysis (Finney, 1971) to determine LC_{50} and IC_{50} values.

RESULTS

Concentrations of 60–300 ppm of *cis*-asarone in artificial diet significantly inhibited the growth of first-instar *P. saucia* ($P < 0.001$, Figure 2) after 10 days. Larvae treated with 120 ppm of the compound weighed approximately 50% of the controls, while at higher concentrations (240–300 ppm), 45–75% mortality was observed. The dietary LC_{50} of *cis*-asarone was calculated as 255 ppm (95% confidence interval: 230–283 ppm). *trans*-Asarone also reduced growth significantly ($P < 0.01$) (Figure 2). After seven days at 300 ppm, larvae weighed 50% of controls. No mortality was observed at any treatment level of *trans*-asarone.

Both *cis*- and *trans*-asarone caused a concentration-dependent reduction in the growth of third-instar larvae that appeared proportional to reduction in food consumption (Figures 3 and 4). *cis*-Asarone caused more severe effects, with 30–100% mortality observed at higher concentrations (1000–2000 ppm).

The efficiency of conversion of ingested food (ECI) in dietary treatments was significantly reduced ($r^2 = 0.91$, $P < 0.05$) by *cis*-asarone in a dose-dependent fashion, as was the efficiency of conversion of digested food (ECD)

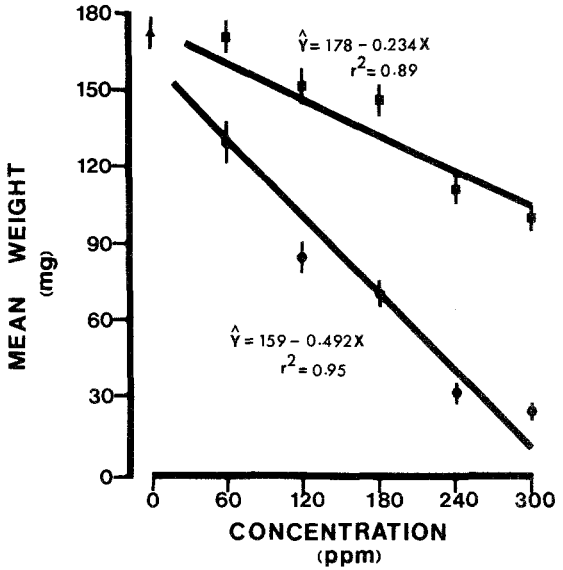

FIG. 2. Relationship between day 10 mean larval weight and dietary *cis*-asarone (●) and *trans*-asarone (■) concentration to *P. saucia* neonate larvae. Error bars represent SEM.

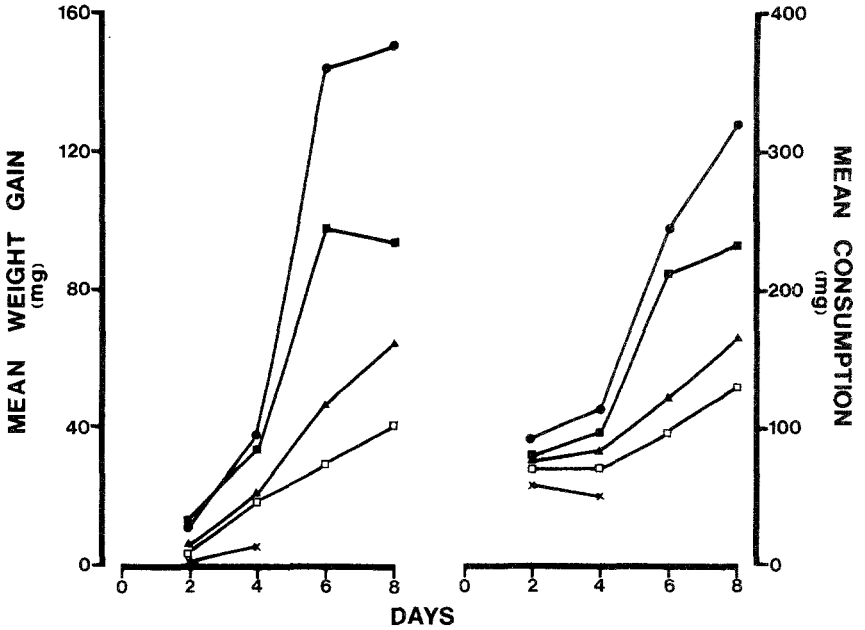

FIG. 3. Concentration-dependent reduction in second-instar *P. saucia* larval weight gain and food consumption at dietary levels of 2000 (x), 1000 (□), 500 (▲), and 250 (■) ppm of *cis*-asarone (controls = ●).

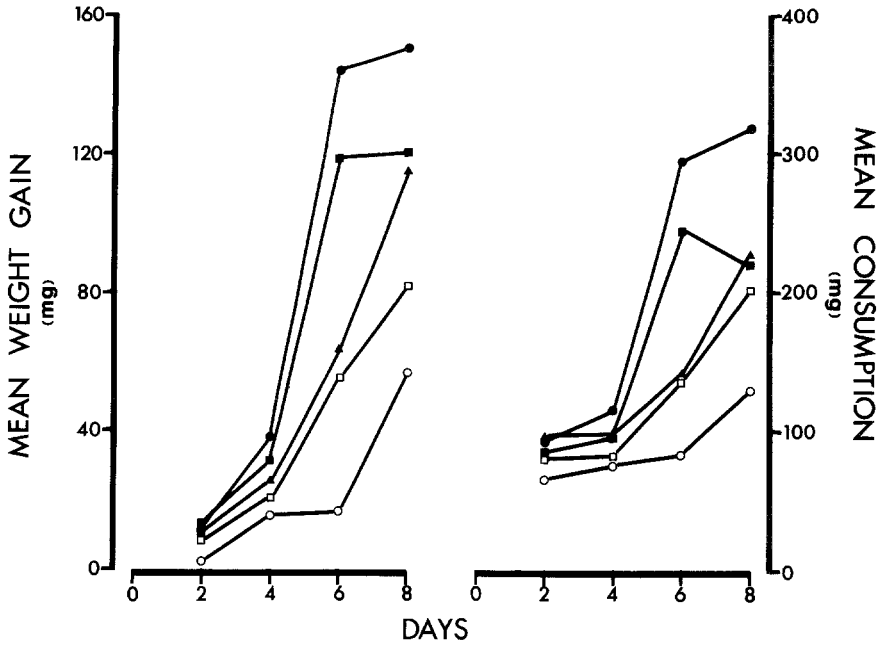

FIG. 4. Concentration-dependent reduction in second-instar *P. saucia* larval weight gain and food consumption at dietary levels of 2000 (○), 1000 (□), 500 (▲) and 250 (■) ppm of *trans*-asarone (controls = ●).

(Table 1). No significant difference in either value was observed from *trans*-asarone dietry treatment ($P > 0.05$, Table 2). Both isomers caused significant reduction in the relative consumption rate (RCRi) ($r^2 = 0.92$ and 0.84 respectively, $P < 0.05$, Tables 1 and 2). In the case of the *trans*-asarone treatments, reduction in RGRi closely paralleled reduction in RCRi. Both isomers, however, caused no reduction in approximate digestibility (AD) of food in the larvae.

The effects of the two asarones on nutritional indices following topical application were more severe for the *cis* isomer, which caused significant decreases in both ECI and ECD ($r^2 = 0.77$ and 0.75 respectively, $P < 0.05$, Table 1). *trans*-Asarone caused no significant reduction in these parameters ($P > 0.05$, Table 2), but did reduce RCRi ($r^2 = 0.88$, $P < 0.05$).

Both isomers exhibited significant antifeedant activity in leaf disk choice-tests ($P < 0.05$, Figures 5 and 6). Fourth-instar larvae were strongly deterred by *cis*-asarone, with 1 $\mu g/cm^2$ causing approximately 27% deterrence and 27 $\mu g/cm^2$ causing 100% deterrence. For fifth-instar larvae 100% feeding inhibition was observed at 45.0 $\mu g/cm^2$ of *cis*-asarone (Figure 6). IC_{50} values for this isomer were calculated as 2.5 $\mu g/cm^2$ for fourth-instar larvae and 4.0 $\mu g/cm^2$ for fifth-instar larvae. *trans*-Asarone gave IC_{50} values of 17.5 $\mu g/cm^2$ and 22.0

TABLE 1. FEEDING, GROWTH, AND DIETARY UTILIZATION BY *P. saucia* FOURTH-INSTAR LARVAE FED ARTIFICIAL DIET CONTAINING *cis*-ASARONE AND BY TOPICAL APPLICATION OF *cis* ISOMER.

	Nutritional indices ($\bar{X} \pm$ SE)				
	RGRi (mg/mg/day)	RCRi (mg/mg/day)	ECI (%)	ECD (%)	AD (%)
Feeding Concentration in diet (ppm)					
0.0	1.71 ± 0.05[a]	5.43 ± 0.38[a]	31.5 ± 1.2[a]	72.1 ± 4.6[a]	43.7 ± 2.2
250.0	1.34 ± 0.10	4.29 ± 0.31	31.3 ± 1.2	61.7 ± 5.0	50.6 ± 3.8
500.0	1.18 ± 0.08	4.36 ± 0.33	27.0 ± 0.9	59.5 ± 4.8	45.4 ± 3.7
750.0	0.79 ± 0.02	3.05 ± 0.28	25.9 ± 0.9	39.5 ± 3.2	65.6 ± 4.2
1000.0	0.49 ± 0.03	2.28 ± 0.17	21.7 ± 0.7	30.1 ± 1.8	72.0 ± 5.8
Topical dose (μg/insect)					
0.0	2.05 ± 0.10[a]	8.30 ± 0.65[a]	24.8 ± 0.9[a]	41.2 ± 3.3[a]	60.1 ± 4.2[a]
5.0	1.08 ± 0.08	6.30 ± 0.42	18.6 ± 0.5	29.1 ± 0.8	63.9 ± 5.0
10.0	1.00 ± 0.07	5.10 ± 0.38	16.6 ± 0.6	24.7 ± 0.6	67.4 ± 4.8
20.0	0.63[b] ± 0.07	4.50 ± 0.26	14.1 ± 0.3	19.2 ± 0.4	73.4 ± 6.5
30.0	0.54[b] ± 0.03	4.10 ± 0.28	12.5 ± 0.2	17.2 ± 0.3	72.9 ± 6.8

[a]Regression performed within each column showed significant linear relationship ($P < 0.05$).
[b]Indicates mortality of 10 and 30%, respectively, at 20 and 30 μg/insect.

TABLE 2. FEEDING, GROWTH, AND DIETARY UTILIZATION BY *P. saucia* FOURTH-INSTAR LARVAE FED ARTIFICIAL DIET CONTAINING *trans*-ASARONE AND BY TOPICAL APPLICATION OF *trans* ISOMER

	Nutritional indices ($\bar{X} \pm$ SE)				
	RGRi (mg/mg/day)	RCRi (mg/mg/day)	ECI (%)	ECD (%)	AD (%)
Feeding concentration in diet (ppm)					
0.0	1.71 ±0.05[a]	5.43 ± 0.38[a]	31.5 ± 1.2	72.1 ± 4.6	43.7 ± 1.2
250.0	1.76 ± 0.08	5.62 ± 0.40	31.2 ± 1.3	72.1 ± 5.0	43.3 ± 3.2
500.0	1.75 ± 0.05	4.68 ± 0.32	32.6 ± 1.8	98.2 ± 8.5	31.7 ± 2.8
750.0	1.41 ± 0.09	4.53 ± 0.37	31.2 ± 1.3	67.9 ± 4.8	45.8 ± 3.8
1000.0	1.09 ± 0.08	3.75 ± 0.42	29.1 ± 1.0	69.5 ± 4.5	41.8 ± 3.0
Topical dose (μg/insect)					
0.0	1.29 ± 0.08[a]	4.74 ± 0.32[a]	27.2 ± 1.1	68.5 ± 4.3	39.7 ± 3.1
5.0	1.30 ± 0.10	4.66 ± 0.33	30.1 ± 1.1	68.5 ± 3.8	43.8 ± 3.2
10.0	1.13 ± 0.05	4.60 ± 0.40	24.5 ± 0.9	56.1 ± 3.7	43.7 ± 3.5
20.0	1.15 ± 0.05	4.01 ± 0.28	28.7 ± 1.2	66.5 ± 4.0	43.2 ± 3.7
30.0	1.12 ± 0.03	3.96 ± 0.30	28.2 ± 0.8	68.0 ± 4.1	41.5 ± 3.9

[a]Regression performed within each column showed significant linear relationship ($P < 0.05$).

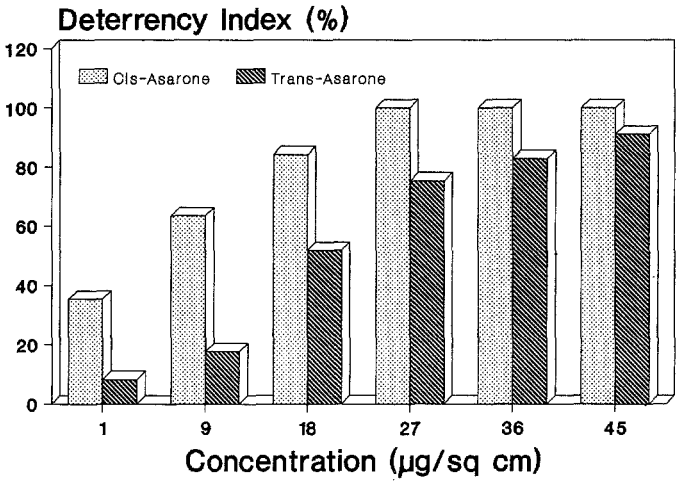

FIG. 5. Percent deterrency to fourth-instar larvae of *P. saucia* at various concentrations of *cis*- and *trans*-asarone in the leaf disk choice test.

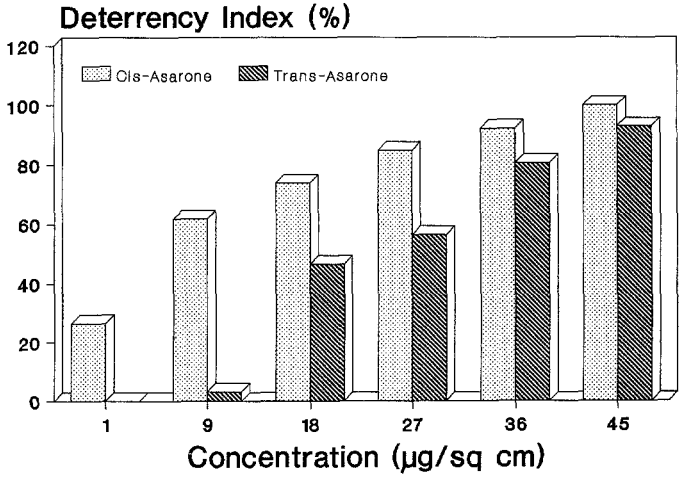

FIG. 6. Percent deterrency to fifth-instar larvae at various concentrations of *cis*- and *trans*-asarone in the leaf disk choice test.

$\mu g/cm^2$ for fourth- and fifth-instar larvae, respectively, with >90% inhibition of both instars at the 45.0 $\mu g/cm^2$ level.

DISCUSSION

The antifeedant and growth inhibitory activities of *A. calamus* oil against *P. saucia* larvae (Koul and Isman, 1990) are attributable to the *cis* and *trans*-asarones present in the oil. *cis*-Asarone, the major component of the oil (ca.

65% by weight in the batch of oil used in our studies), has both antifeedant and growth inhibitory activities, and the toxicity of this compound may contribute to reductions in relative consumption rate. This conclusion is supported by the topical application experiments where *cis*-asarone resulted in lower RGRi vis-à-vis the reduction in ECI and ECD, whereas *trans*-asarone only affected RCRi. The direct toxicity of the *cis* isomer (Janzen et al., 1977; Matolcsy et al., 1981; Ramos-Ocampo and Hsia, 1986) and its antigonadal effects (Koul et al., 1977a,b; Koul, 1979) have been well described in other insects. In spite of the known toxic action of this compound in some species, *cis*-asarone has been shown, surprisingly to be an attractant to oriental fruit flies (Jacobson et al., 1976), and *trans*-asarone acts as an oviposition stimulant for the carrot rustfly (Stadler and Buser, 1984).

In *P. saucia*, *trans*-asarone appears to be an antifeedant alone, lacking the general toxicity of the *cis* isomer. It is apparent that the antifeedant action, at least in the case of the *trans* isomer, is independent of toxicity as no discernible reductions were observed in ECI and ECD, yet the antifeedant action is well demonstrated by the results of direct bioassay for feeding deterrence in the leaf disk choice test. This antifeedant action resembles that of isoasarone (Figure 1) isolated from Japanese piper, *Piper futokadzura*, which deters feeding of *Spodoptera litura* F. larvae at a concentration of 1.0% in leaf disk tests (Matsui et al., 1976). Both of these compounds are similar in structure, differing only in the position of the double bond in the allyl side chain (Figure 1). This suggests that the toxicity of *cis*-asarone is due to the *cis* configuration rather than to the position of the double bond. It has already been established that changes in the phenyl substituents influence the activity of asarones considerably (Saxena et al., 1977).

It can be concluded that asarones found in *A. calamus* oil have different bioactivities towards insects. While *cis*-asarone is responsible for antigonadal activity in some species (Koul, 1979), it has strong growth- and feeding-inhibitory effects in others. In contrast, *trans*-asarone does not induce any toxic effects but acts solely as a feeding deterrent similar to isoasarone (Matsui et al., 1976).

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ENANTIOMERIC SYNTHESIS OF DOMINICALURE, AGGREGATION PHEROMONE OF LESSER GRAIN BORER, *Rhyzopertha dominica* (F.)[1]

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Abstract—(*S*)-(+)-1-Methylbutyl (*E*)-2-methyl-2-pentenoate, **1**, and (*S*)-(+)-1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate **2**, the aggregation pheromone for lesser grain borer *Rhyzopertha dominica* (F). were synthesized from crotylaldehyde in an overall yield of 30%. The chiral intermediate **6** was prepared in 90% enantiomer excess, employing the Sharpless asymmetric epoxidation.

Key Words—Lesser grain borer, *Rhyzopertha dominica* (F), Coleoptera, Bostrichidae, aggregation pheromone, chiral synthesis.

INTRODUCTION

The lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera, Bostrichidae) is an important pest of stored products all over the world, causing serious damage (Agriculture Press, 1979). The aggregation pheromone of this insect was isolated and recently identified as a mixture of **1** and **2** named as dominicalure (Williams et al., 1981). Both **1** and **2** have a chiral center with *S* configuration. Although **1** and **2** were reportedly synthesized from a natural amino acid in eight steps, the method was unsatisfactory and suffered from low overall yield (Williams et al., 1981). Therefore an effort was made to improve the synthesis of **1** and **2**. We report the synthesis of the enantiomers of **1** and **2** (Schemes 1 and 2) employing modified Sharpless asymmetric epoxidation (Wang et al., 1985).

[1] Part of this work was presented in the China-Japan Seminar on Insect Semichemicals, Institute of Beijing Zoology, Academia Sinica, Beijing, China, 1988.

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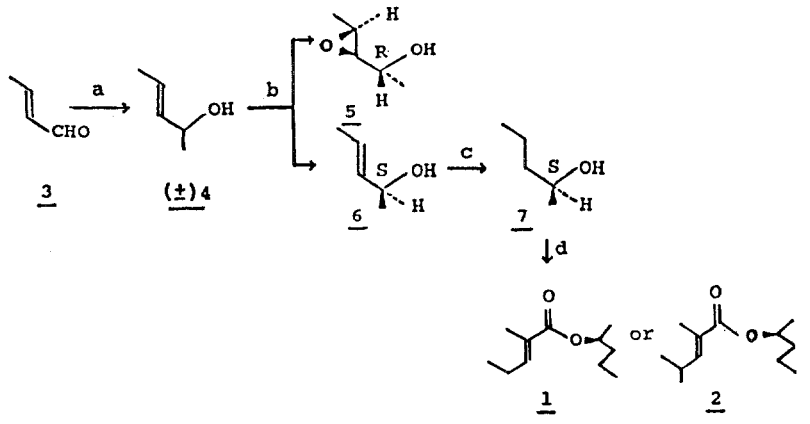
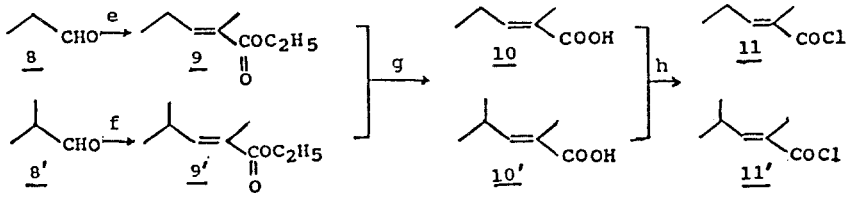


SCHEME 1. Reaction conditions for schemes 1 and 2: a. CH_3MgBr/ether; b. D-(−)-DIPT, *t*-BuOOH, $Ti(OPr^i)_4$CaH-Silica-gel; c. Pd/C, H_2; d. **11** or **11′**/hexan; e. $Ph_3PCH(CH_3)COOC_2H_5Br$, $CH_3ONa-CH_3OH$; f. $Ph_3PCH(CH_3)COOC_2H_5Br$, NaH—DMSO—THF; g. NaOH—H_2O; h. $SOCl_2$.



SCHEME 2.

The overall yields were increased to 30%. 3-Penten-2-ol (±) **4** (Coburn, 1955), prepared from 2-butenal **3** and methyl magnesium bromide, was exposed to CaH and a silica gel-catalyzed Sharpless reagent system [D-(−)-diisopropyltartrate, *tert*-butyl-hydroperoxide and titanium tetraisopropoxide] under kinetic resolution conditions to give an optically active alcohol (*S*)-(+)-**6** in 90% ee along with an optically active epoxy alcohol (2*R*, 3*R*, *R*)-**5**, which can be used for other purposes. Reduction of **6** to **7** with H_2 over palladium catalyst followed by esterification of **7** with **11** or **11′** produced **1** and **2**, respectively.

SYNTHESIS

All boiling points are uncorrected. IR spectra were recorded on a Perkin-Elmer 577 Spectrometer. MS was performed on Finnigan 4020. GC-MS was conducted on QP-1000 (Shimadzu). [^{1}H]NMR spectra were determined on EM-360L or XL-200 using tetramethylsilane as the internal standard. GC analyses

were carried out on a Shanghai Analytical Instrument Factory GC-103 and on a HP-5880 equipped with capillary columns (SE-54, 50 m × 0.2 mm). Optical rotations were measured on Rudolph Research Autopol III.

(±)-(E)-3-Penten-2-ol **4**. To stirred, freshly prepared methyl magnesium bromide (0.5 mol) in ether (150 ml) was added slowly a solution of 2-butenal (35 g, 0.5 mol) in ether (50 ml) at room temperature. The mixture was refluxed for 1 hr. A saturated aqueous solution of ammonium chloride was added to the mixture under ice cooling, followed by stirring for an additional hour. Work-up in the usual manner provided **4** (36.5 g), bp 119–121°C, in 85% yield. IR, ν_{max}: 3320, 1675 cm^{-1}. [^{1}H]NMR, δ: 1.18–1.22 [3H, d, J = 7.2, C(OH)C$\underline{H}_3$], 1.61–1.64 (3H, d, J = 5.4, C=C−CH_3), 3.98–4.2 [1H, br, C=CC$\underline{H}$(OH)], 5.55–5.62 (2H, m, CH=CH) ppm. MS, *m/z*: 86(M, 4.6), 41(CH_3CH=CH, 100).

(S)-(+)-(E)-3-Penten-2-ol **6**. Compound **4** (8.5 g, 100 mmol) was injected into a mixture of Ti(Oi-Pr)$_4$ (6 ml, 20 mmol), D-(−)-DIPT (4 ml, 20 mmol) CaH (60 mg, 1.5 mmol), and silical gel (80 mg, 1.3 mmol) in dried CH_2Cl_2 (300 ml) at −40°C under N_2 flow. The mixture was stirred for 20 min, followed by injection of *t*-BuOOH (60 mmol, 5.92 M). The reaction was monitored by TLC until a mixture of equal amount of the epoxide **5** and alcohol **6** was produced. The reaction was quenched by addition of 10% tartaric acid in water (80 ml) at −20°C and stirred at room temperature until the aqueous layer became clear. The mixture was extracted with CH_2Cl_2 five times. Concentration of the extractant gave a crude product, which, after column chromatography over silica gel, offered **6** (2.76 g) in 60% yield, bp 119–121°C. The optical purity of (*S*)-(+)-**6** was estimated to be 90% by GC analysis of its MTPA ester [(*S*)-α-trifluoromethyl-α-methoxyphenylacetate] (Dale, 1969). (*S*)-(+)-**6** showed the same spectral data as **4**.

(S)-(+)-Pentan-2-ol-7. Hydrogenation of (*S*)-(+)-**6** (1.72 g, 20 mmol, in hexane (30 ml) over 10% Pd/C (0.2 g) yielded (*S*)-(+)−**7**, which was sufficiently pure to be used in the next step without further purification. GC analysis (10% FFAP 2 m, 79°C, N_2: 40 ml/min) R_t: 4.0 min for **7** and 6.2 min for **6**.

Ethyl-(E)-2-methyl-2-pentenoate **9**. A mixture of [(ethyl acetoxy) methyl]triphenylphosphonium bromide (8 g, 14 mmol), prepared according to Isler et al. (1957), and 2 N sodium methoxide-methanol (8 ml) in dichloromethane (60 ml) was stirred under N_2 at 50°C for 1 hr. Aldehyde **8** (0.4 g, 6.7 mmol) was then added to the freshly formed Wittig reagent, and the mixture was refluxed for 8 hr. The solid was filtered out, and the filtrate was washed with water. The filtrate was concentrated to give a crude oil, which, after flash column chromatography, gave **9** (817 mg) in 86% yield. The geometric purity of **9** was shown to be 99% *E* by GC analysis. IR, ν_{max}: 2900, 1700, 1640 cm^{-1}. [^{1}H]NMR, δ: 0.98 [3H, t, J = 7.5, CH_3], 1.19 [3H, t, J = 7.5, CH_3], 1.7 [3H, s, =CCH_3], 1.92–2.19 (2H, m, CH_2C=), 3.8–4.28 (2H, q, J = 7.5,

OCH_2), 6.40–6.73 (H, t, J = 7.5, −CH=C) ppm. MS, m/z: 142 (M^+, 1.39), 129(26.5), 97(20.2), 69(77.6).

(E)-Methyl-2-pentenoic acid ***10***. Hydrolysis of **9** (56 mg, 0.39 mmol) was accomplished with 2 N aqueous NaOH (5 ml) at room temperature for 5 hr. The reaction mixture was acidified with 2 N HCl, White needle crystals were collected, and washed with water to give **10** (40 mg) in yield of 90%. IR, ν_{max}: 2900, 1700cm^{-1}. [^{1}H]NMR, ν: 1.00(3H, t, J = 7, CH_3), 1.76(3H, s, C=CCH_3). 2.11–2.49 (2H, m, CH_2), 6.25–6.50 (1H, t, J = 7, C=CH), 11.06 (1H, s, COOH) ppm. MS, m/z: 114(M^+, 32.9), 85 [CH=C(CH_3)COOH, 4.26], 69(82.9), 41(100).

1-Methylbutyl-(E)-2-methyl-2-pentenoate . A mixture of **10** (152 mg, 1.33 mmol) and thionyl chloride (150 mg, 1.5 mmol) was heated at 50°C for 20 min, and then the excess thionyl chloride was removed in vacuo. The chloride so formed was treated with (*S*)-(+)-**7** (176 mg, 2.0 mmol) at 50°C for 30 min. Work-up in the usual manner yielded **1** (159 mg) in 65% yield. $[\alpha]_D$ = +14.2° (c = 1.265, ether). Lit $[\alpha]_D$ = +13.4° (c = 0.175, ether) (Williams, 1981). IR, ν_{max}: 2900, 1700, 1640 cm^{-1}. [^{1}H]NMR, δ: 0.90–1.10 (6H, 2t overlap, J = 7.5, $2CH_3$), 1.18–1.26 (3H, d, J = 7.4, CH_3), 1.28–1.60 (4H, m, CH_2CH_2), 1.80 (3H, d, J = 1.2, C=CCH_3), 2.05–2.21(2H, bq, J = 7.5, C=CCH_2), 4.82–5.04 (1H, q, J= 7.5, COOCH), 6.59–6.76 (1H, dt, J = 1.5, 7.2, CH=C) ppm. MS, m/z: 184 (M^+, 0.3), 115(73.6), 97(100). HRMS, m/z: calc. for $C_{11}H_{20}O_2$ (M−1): 184.267, found: 184.269; Calc. for $C_6H_{11}O_2$ (M−C_5H_{10}): 115.151, found: 115, 156.

Ethyl-(E)-2,4-dimethyl-2-pentenoate ***9′***. A mixture of isobutyaldehyde (0.72 g, 0.01 mmol), **8′** (12 g, 0.021 mmol), sodium hydride (0.36 g, 0.015 mmol), dimethyl sulfoxide (5 ml), and tetrahydrofuran (30 ml) was refluxed under N_2 flow for 10 hr. Work-up in the same manner as that of **9** gave **9′** (1.43 g) in yield of 92%. IR, ν_{max}: 2900, 1700, 1640, 1350 cm^{-1}. [^{1}H]NMR, δ: 1.0–1.3 (9H, m, $3CH_3$), 1.75(3H, s, CH_3), 2.4–2.7(1H, br, CH=C), 3.9–4.3 (2H, q, $COOCH_2$), 6.3–6.5 (1H, d, J = 10, CH=C) ppm. MS, m/z: 156(M, 4.6), 55(100), 83(81.4), 111(54.0), 127(14.5).

(E)-2,4-Dimethyl-2-pentenoic acid ***10′***. Compound **10′** was prepared in the same manner as that of **10** in yield of 87%. IR, ν_{max}: 2900, 1700, 1640, 1350 cm^{-1}. [^{1}H]NMR, δ: 1.1(6H, d, J = 7.2, $2CH_3$), 1.8 (3H, s, C=CCH_3), 2.86 (1H, m, C<u>H</u>CH=C), 6.7–6.85 (1H, d, J = 7.2, CH=C−), 11.8(1H, s, COOH) ppm. MS, m/z: 128(M+4.1), 83(79.6), 85(100).

1-Methylbutyl (E)-2,4-dimethyl-2-pentenoate . In the same manner as that of **1**, **2** (129.9 mg) was prepared from **6** (176 mg, 2.0 mmol) and **10′** (128 mg, 1 mmol) in yield of 65.5%. $[\alpha]_D$ = +10.9 (c = 1.256, ether). IR, ν_{max}: 2900, 1700, 1300 cm^{-1}. [^{1}H]NMR, δ: 0.9 (3H, t, CH_3), 1.96, and 2.04 (6H, d, J = 7.5, $2CH_3$), 1.22–1.50 (4H, m, CH_2CH_2), 1.19–1.23 (3H, d, J = 7.4, $COOCCH_3$), 1.88(3H, d, J = 1.5, C=CCH_3), 2.44–2.66(1H, m, C=CCH), 4.81–5.08(1H, m, COOCH), 6.42–6.48(1H, dd, J= 10.8, 1.5, C=CH) ppm.

MS, *m/z*: 199 (M + 1, 10.5), 129(100), 111(96.74), 70(30.56) HRMS, *m/z*: calc. for $C_{12}H_{22}O_2$ (M + 1): 199.3046, found: 199.3120; calc. for $C_7H_{11}O_2$: 127.1648, found: 127.1796.

DISCUSSION

The key step as shown in Scheme 1 is the kinetic resolution of the racemic allylic alcohol (±)-**4** via the modified Sharpless epoxidation. The reaction was quenched with 10% tartaric acid when 50–55% conversion was reached, giving a 65% yield of (*S*)-(+)-**6**, 90% ee by TLC or GC. The yield was calculated based upon the half conversion.

High volatility and moderate water solubility of **6** made it difficult to handle such a compound. Therefore, more extraction with a low boiling solvent is needed to prevent the loss of the chiral product during isolation.

Treatment of the aldehyde **8** with Wittig reagent under sodium methoxide-methanol offered **9** in 86% isolated yield. In the case of **8′**, the reaction failed under the same conditions in leaving **8′** untouched, possibly due to the steric hindrance of the 2-methyl group in **8′**. We found that the reaction went smoothly when sodium hydride, DMSO, and THF were used, giving a 92% yield of the product.

Bioassays of dominicalure **1** and **2** are underway, and the results will be presented elsewhere.

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DEVIL'S-CLAW (*Proboscidea louisianica*), ESSENTIAL OIL AND ITS COMPONENTS
Potential Allelochemical Agents on Cotton and Wheat

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Abstract—The potential allelopathic activity of devil's-claw [*Proboscidea louisianica* (Mill.) Thellung] essential oil and a few of the compounds it contains on the elongation of cotton (*Gossypium hirsutum* L.) and wheat (*Triticum aestivum* L.) radicles was studied using a Petri dish bioassay. Essential oil was collected by steam distillation using an all-glass–Teflon assembly. Ether extracts of the steam distillates from fresh devil's-claw were inhibitory to cotton and wheat radicle elongation. The following six components of devil's-claw essential oil identified by CGC-MS-DS were inhibitory to cotton and/or wheat at a concentration of 1 mM: vanillin, piperitenone, δ-cadinene, *p*-cymen-9-ol, α-bisabolol, and phenethyl alcohol.

Key Words—Allelopathy, phytotoxic, allelochemical, α-bisabolol, δ-cadinene, *p*-cymen-9-ol, essential oil, germination, phenethyl alcohol, piperitenone, vanillin, *Gossypium hirsutum*, *Proboscidea louisianica*, *Triticum aestivum*.

INTRODUCTION

Devil's-claw, *Proboscidea louisianica*, formerly called unicorn plant and devils-claw, is a member of the Martyniaceae and is native to the southwestern United States and northern Mexico (Martin and Hutchins, 1980). It is sometimes cultivated for its young pods, which are pickled, and for mature pods used as

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ornaments and in basketweaving (Nabhan et al., 1981). Devil's-claw is a spreading annual with prostrate branches spanning up to 1 m and is densely covered with clammy, articulate, glandular hairs, which gives the plant a very oily appearance and a strong, musty odor (Brooks and Weedon, 1986).

Seed germination of devil's-claw is erratic, and the sseds often show extreme dormancy when freshly harvested (Heit, 1971; Riffle et al., 1988a). The best germination occurred when both the outer black leathery seed coat and the inner, papery white membrane were removed (Heit, 1971; Cooley et al., 1973; Riffle et al., 1988a). Gibberellic acid (GA_3) greatly increased germination but was inhibitory to subsequent growth (Anderson, 1968).

Many weedy pests in the cotton-growing areas of Oklahoma and West Texas are responsible for cotton yield reductions (Rushing et al., 1985a,b; Mercer et al., 1987). Earlier research on devil's-claw interference with cotton showed that cotton lint yield was reduced 83% in weed-infested areas (Cooley et al., 1973). Bridges and Chandler (1984) reported a lint yield reduction of 34% when the weed population density was 4 plants/6 m of row. Mercer et al. (1987) found reductions of 20% when the weed density was only 1 plant/10 m of row; cotton plant height was reduced 43% and lint yield was reduced up to 74% when the weed density was 32 weeds/10 m of row.

Much of the research in allelopathy has centered on crop-weed associations. Results of such research have identified many specific cases of biochemical interactions between the crop and weed. Tuber extracts and residues of yellow nutsedge (*Cyperus esculentus* L.) reduced the growth of corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] (Drost and Doll, 1980). Plant residue and ethanolic extracts of Canada thistle [*Cirsium arvense* (L.) Scop.] were inhibitory to barley (*Hordeum vulgare* L.) and cucumber (*Cucumis sativus* L.) radicle elongation (Stachon and Zimdahl, 1980). Volatile methyl ketones and low-molecular-weight aliphatic alcohols and aldehydes have been isolated and identified from Palmer amaranth (*Amaranthus palmeri* S. Wats.) residues and these were reported to be allelopathic to onion (*Allium cepa* L.), carrot (*Daucus carota* L.), tomato (*Lycopersicon esculentum* Mill.), and Palmer amaranth itself (Bradow and Connick, 1988a,b). A glycoside of molecular weight 460 was isolated from the rhizomes of quackgrass [*Agropyron repens* (L.) Beauv.] by methanol–water extraction, purified, and proved inhibitory to the seedling root growth of corn, oat (*Avena sativa* L.), cucumber, and alfalfa (*Medicago sativa* L.) (Gabor and Veatch, 1981). Much allelopathic research has concentrated on compounds that are leached from plant litter or produced by plant decomposition.

Some allelochemicals can be released from plants into the air and soil because of their low molecular weight and relatively high vapor pressure. The inhibitory volatile chemicals that emanate from the leaves of *Salvia leucophylla* (Muller et al., 1964; Muller and del Moral, 1966) were found to include cineole

and camphor, which were highly inhibitory to root and hypocotyl growth in germinating herb seeds (Muller et al., 1968). Phytotoxic volatiles were found to be associated with the weed Palmer amaranth; one of the compounds identified was 2-heptanone, which was strongly inhibitory to the germination of onion and carrot at concentrations of 1 ppm (Connick et al., 1987).

Preliminary research demonstrated that aqueous extracts of various parts of devil's-claw were inhibitory in Petri dish bioassays to radicle elongation of cotton, wheat, and even devil's-claw itself (Riffle et al., 1988a). Furthermore, since devil's-claw is oily and odoriferous when growing, we investigated possible phytotoxic effects caused by its volatiles. Therfore, the objectives of this research were to isolate the essential oil of devil's-claw and test it and some of its components for phytotoxic activity. A preliminary account of this research has been presented and published in abstract form (Riffle et al., 1988b, Waller et al., 1987, Waller, 1989).

METHODS AND MATERIALS

Plant Materials. Devil's-claw was collected on August 27 and September 10, 1986, at Perkins, Oklahoma, and separated into roots, stems and leaves, and pods. The plants collected in September were in the early stages of senescence. Fresh weights of the plant tissues collected on August 27 were 1.7 kg of stems and leaves, 3.7 kg of pods, and 0.2 kg of roots, and collected on September 10 were 1.9 kg of stems and leaves, 4.3 kg of pods, and 0.2 kg of roots. In 1987, devil's-claw was collected in an active growth stage on August 14, 22, and 27. Approximately 5 kg of plant material were collected on each date by cutting the stems at ground level. Plant material was not separated in 1987. In all cases, the material was taken immediately to the laboratory for steam distillation.

Steam Distillation. Steam distillation was used to isolate the devil's-claw essential oil. The steam distillation apparatus was an all-glass assembly except for Teflon stopcocks and sleeves. Distillations from normal and acidic suspensions of all above ground plant parts were made in 1986 by loading the plant parts separately into a 10-liter round-bottom flask and steam distilling for 5 hr. The condensate collected (approximately 3 liters) was saturated with 1.1 kg NaCl and extracted with three 1-liter portions of ethyl ether; the extract was dried over anhydrous Na_2SO_4 and evaporated under nitrogen. The residue suspension left after distillation was acidified with 1 liter of 2 N HCl to a pH of 0.8 and redistilled for 5 hr. The condensate was processed in the same way as in the normal distillation. In 1987, only normal steam distillations were made of the whole plant (minus the roots) in 5-kg batches.

Capillary gas chromatography. The initial capillary gas chromatographic (CGC) runs were carried out on a Hewlett Packard model 5880 gas chromatograph containing a flame ionization detector and an OV-1 fused silica column 50 m × 0.32 mm. The samples were taken up in ether and analyzed using a 1.5-μl injection with a splitter ratio of 25 : 1, with a He flow of 0.5 ml/min, and the oven at 50°C, programmed at 2°C/min to 225°C and held there for 60 min.

CSG-MS-DS Analysis. The essential oil derived from the roots by normal distillation, and that obtained from pods in aqueous acid, were analyzed with a capillary gas chromatography–mass spectrometry–data system (CGC-MS-DS) using a Kratos MS-50 mass spectrometer with a resolution of 2000. The MS-50 was equipped with a Varian model 3700 gas chromatograph containing an OV-1 fused-silica column, 50 m × 0.32 mm. The oven temperature was programmed to rise at 2°C/min from 50°C to 225°C and be held there for 60 min, with a He flow of 0.5 ml/min. The data were acquired and analyzed with a modified Kratos DS-55 data system (Bondarovich et al., 1987). Identifications were based on comparison of known with unknown spectra, visual interpretation of the fragmentation patterns, and the IFF proprietary indices of the elution program, which are similar to Kovats indices. The CGC-MS-DS and the mass spectra are not shown, but are published in a dissertation by Riffle (1988).

Bioassays. In 1986, 18 ml of methanol was used to dilute the residues from the ether extracts of both the normal and acidic steam distillates. These solutions were added in 2-ml portions to 9.5-cm Petri dishes containing two layers of 9-cm Whatman No. 1 filter paper, and the methanol was allowed to evaporate for approximately 2 hr. Ten cotton or wheat seeds were then placed between the filter paper layers, 3 ml of distilled water was added, and the covered dish placed in a sealed polyethylene bag to prevent further loss of volatiles. In 1987, the essential oil collected from devil's-claw during August, and selected reference compounds (δ-cadinene and *p*-cymen-9-ol, International Flavors and Fragrances; α-bisabolol and piperitenone, Firmenich Inc., Princeton, New Jersey 08543; and vanillin and redistilled phenethyl alcohol, Sigma Chemical Co., St. Louis, Missouri 63178), were dissolved in methanol to produce 1 mM concentrations. Cotton was used because it is the usual crop in this area that grows in close association with devil's-claw. Wheat was included because it is frequently planted as a rotational crop with cotton.

To make the 1 mM concentration of devil's-claw essential oil in methanol it was assumed that the average molecular weight was 200. The bioassays were conducted in the same way as those in 1986, except that in the 1987 essential oil bioassay 4 ml of the 1 mM solutions were added to the Petri dish, and only wheat was added as a bioassay species. All experiments were designed as a randomized complete block (Montgomery, 1984) with each of four replications comprising a tray level in the germinator. The germination temperature was

27°C for cotton and 20°C for wheat. After the seeds had been allowed 72 hr for germination and growth, measurements of radicle length were made.

Statistical Analysis. All bioassays consisted of two runs with four replications. Before data analysis, the variances of all treatments were tested by using the VARCOMP procedure in SAS Statistical Analysis Systems, SAS Institute Inc., Cary, North Carolina 27511. Generally, if the treatment inhibited radicle elongation, the variance for that treatment was smaller than for treatments without inhibitor. Therefore, the variances were determined and weighted before analysis. After analysis, no run-by-treatment interaction was detected for any of the bioassays, so data obtained in the runs were pooled and analyzed. The treatment means were separated using the protected least significant difference (LSD) at the 0.05 level of probability.

RESULTS AND DISCUSSION

Response of Cotton to Devil's-claw. An understanding of the cause-and-effect relationship in allelopathy or specific allelochemicals requires knowledge of how the chemicals are emitted from the producer (devil's-claw) and transported to the target plant (cotton). Devil's-claw, when growing with cotton, can cause substantial yield reductions (Mercer et al., 1987), as already noted. The plant is densely covered with glandular hairs, each tipped with a droplet of oil. A strong acrid odor of the plant growing in the field can be detected, especially to windward. Figure 1 shows that the cotton plant can be deleteriously affected by contact with the devil's-claw volatiles (Riffle et al., 1988b). We suggest that components of the essential oil can make contact with other plants in the vicinity, especially cotton. Moreover, during the night when the temperature is lower, the essential oil components can blanket the area and affect the cotton deleteriously. Thus these volatiles could be responsible for some of the phytotoxic activity. Senescence and, finally, death occur in a portion of a cotton leaf exposed to a devil's-claw leaf only a few centimeters away (when the wind blows, actual physical contact can be made between the leaves). Some cotton plants show little or no response to the devil's-claw plant, indicating that they can rapidly metabolize volatile compounds produced by the weed.

A possible sequence of events associated with senescence and ultimately death of plant cells is described by Woodhouse (1987). The pathway probably involves the failure of a series of functions. According to Woodhouse, the synthesis of some RNA and protein occurs right up to the later stages of leaf senescence; physiological stress (as indicated by declining photosynthetic activity), but retention of mitochondrial integrity, respiratory activity, plastid envelope, and leaf turgor are maintained until the very last stages of senescence, when

FIG. 1. Photograph of cotton leaf and devil's-claw leaves and flowers in the field near Perkins, Oklahoma, July 1987. (Note that the necrotic and senescent tissue is only on the cotton.)

the leaf is completely yellow. Thus the probable sequence involves the preservation to a late stage of structure required for the translocation of the products of degradation to other living parts of the cotton plant.

Steam Distillation. The ether-free extracts of the steam distillates were viscous, dark yellow to light brown oils with a pungent, musty odor and recovery was generally higher for the August 27 distillation (Table 1). The devil's-claw was in active growth at this time and was geen and succulent. The amount of oil obtained from the September 10 distillation was lower, and the devil's-claw was in early senescence. The weights of the oils obtained by steam distillation in 1987 were 61 mg on August 18, 549 mg on August 22, and 76 mg on August 27 (0.0012%, 0.0110%, and 0.015% of fresh weight, respectively), which was equal to or more than the essential oil yield in 1986.

Distillation of devil's-claw plant material (residues) with 2 N HCl (which followed the normal steam distillation) was done because of the results of Waller and Johnson (1984) with *Nepeta cataria* L. (oil of catnip or catmint), which liberated four times as much steam-volatile material containing stereoisomeric dihydronepetalactones and indicated additional metabolically active compounds. The result was little change in the quantity and composition of devil's-claw essential oil recovered, but they showed some differences in biological

TABLE 1. AMOUNT OF ESSENTIAL OIL RECOVERED FROM STEAM DISTILLATION OF DEVIL'S-CLAW IN 1986

Distillation	Sample date: August 27 — Essential oil recovered (mg)	August 27 — Percent of fresh weight (%)	September 10 — Essential oil recovered (mg)	September 10 — Percent of fresh weight (%)
Normal				
Leaves and stems	57	0.0034	4	0.0002
Pods	40	0.0011	16	0.0004
Roots	9	0.0047	14	0.0070
Acidified				
Leaves and stems	47	0.0028	23	0.0012
Pods	11	0.0003	13	0.0003
Roots	14	0.0069	15	0.0076

activity. The acidification releases compounds otherwise bound to the plant residue.

CGC-MS-DS Analysis. Obtaining the MS-50 profile of the essential oils required approximately 140 min and 3500 spectra for each sample; the results indicated that 150–220 compounds were present. From this complex mixture, peaks corresponding to the following compounds were identified from the normal steam distillate of the roots in order of appearance from the CGC column: *p*-vinylphenol, piperitenone, vanillin, 2-methyl-1,4-naphtoquinone, ionol, 1, 3, 5-tri-*tert*-butylbenzene, α-bisabolol, hexadecanoic acid, traxolide, δ-cadinene, and 1-hydroxy-(2 (or 3)-hydroxymethyl)-9, 10-anthracenedione. A trace of 6-methyl-5-hepten-2-one was found. The remaining compounds were mostly terpenoids, terpenes, and other hydrocarbons and were not identified. The following peaks were identified in the steam distillate from the HCl-treated pods: phenylethyl alchohol, a trimethylcyclohexanone, *p*-cymen-9-ol, vanillin, 2-ethylbenzimidazole (tentatively), and dodecanoic and hexadecanoic acids. The remaining peaks were due mostly to sesquiterpenes and other hydrocarbons and again were not identified.

Bioassays. In 1986, the normal essential oils collected on August 27 from the leaves and stems, from the pods, and the essential oil of the HCl-treated pods were inhibitory (27, 37, and 15%, respectively) to cotton radicle elongation (Table 2). In this bioassay, the essential oil recovered was diluted with methanol to make 18 ml, so the concentrations were different between extracts. High concentrations of the normal essential oil of the leaves and stems, and of

TABLE 2. EFFECTS OF ESSENTIAL OILS OF DEVIL'S-CLAW COLLECTED ON AUGUST 27 AND SEPTEMBER 10 ON COTTON RADICLE LENGTH

	Date of collection and distillation[a]			
	August 27		September 10	
Distillation	Concentration (mg/dish)	Inhibition (%)	Concentration (mg/dish)	Inhibition (%)
Control (methanol)		0a		0a
Normal				
Leaves and stems	3.18	27b	0.22	2a
Pods	2.22	37b	0.90	10a
Roots	0.52	8a	0.78	0a
Acidified				
Leaves and stems	2.60	7a	1.28	33b
Pods	0.62	15b	0.72	11a
Roots	0.76	8a	0.84	12a

[a] Means followed by the same letter are not significantly different at the 5% level using the protected LSD.

the pods, were present in the Petri dish, and these were highly inhibitory. However, inhibition was not entirely dependent of concentration. The essential oil obtained from HCl-treated leaves and stems was not as inhibitory as that of the HCl-treated pods, even though the latter was present in much less concentration (2.60 vs. 0.62 mg/dish). The essential oils collected from pods by the normal and acidic steam distillations were the most inhibitory. Essential oils from the roots, whether produced from normal or acidic environments, did not significantly inhibit cotton radicle elongation when compared to the control.

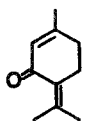
Of the steam distillations conducted on the September 10 samples, only the essential oil of the HCl-treated leaves and stems decreased cotton radicle elongation by as much as 33% (Table 2). Since the plants were beginning to senesce, a buildup of other compounds could have been responsible for the increased inhibition, but the oil was not analyzed. The "normal" essential oils were less concentrated in this bioassay and were not significantly inhibitory.

After analysis of the essential oil in 1987, several authentic compounds were obtained. Of solutions (1 mM) of 13 such compounds in methanol, six were inhibitory to cotton and five to wheat (Table 3, Figure 2). Of the six compounds, three were identified in the root, two in the pods, and one (vanillin) in both the root and the pods; however, note that root oil was not inhibitory to cotton. Of these six inhibitory compounds, four are terpenoid in nature: piperitenone and *p*-cymen-9-ol (Figure 2, a and b) are monoterpenes, and α-bisabolol and δ-cadinene (Figure 2, d and f) are sesquiterpenes. Some monoterpenes

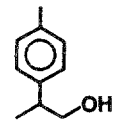
TABLE 3. COMPOUNDS TESTED AT 1 mM CONCENTRATION FOR PHYTOTOXIC ACTIVITY ON COTTON OR WHEAT RADICLE ELONGATION[a]

Compound	Plant part extracted	Relative amount	Cotton	Wheat
α-Bisabolol	root	moderate	+	+
δ-Cadinene	root	moderate	+	+
p-Cymen-9-ol	pod	low	+	+
6-Methyl-5-hepten-2-one	root	low	−	NT
2-Methyl-1,4-naphthoquinone	root	moderate	−	−
Phenethyl alcohol	pod	low	+	+
Piperitenone	root	low	+	+
2,4,4-Trimethylcyclohexanone	pod	high	−	NT
Vanillin	root, pod	high	+	−
p-Vinylphenol	root	low	−	NT

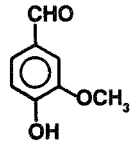
[a] + indicates visually observable inhibition, − indicates no visually observable inhibition, NT = not tested.



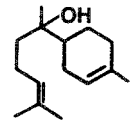
a. piperitenone (root)



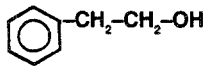
b. *p*–cymen–9–ol (pod)



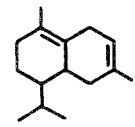
c. vanillin (root, pod)



d. α–bisabolol (root)



e. phenethyl alcohol (pod)



f. δ–cadinene (root)

FIG. 2. Components of devil's-claw essential oil that were inhibitory to cotton and/or wheat at 1 nM concentration. Compounds are listed from most inhibitory to least inhibitory.

are known to be inhibitory (Asplund, 1968, 1969; Muller et al., 1968; Muller and del Moral, 1966; Fischer, 1986). Asplund (1969) reported that camphor, pulegone, and borneol were extremely toxic to radish (*Raphanus sativus* L.) and wheat and stated that monterpenes are among the most phytotoxic compounds produced by plants. Sesquiterpenes, such as β-bisabolene, isolated from common ragweed (*Ambrosia artemisiifolia* L.) caused strong inhibition of germination of onion, oats, and ryegrass (*Lolium multiflorum* Lam.) (Fischer, 1986).

Phenolic compounds, such as vanillin (Figure 2, c), have been implicated as being allelopathic agents released as root exudates and from decomposing plant litter (Wang et al., 1967). In the mitochondria, phenolic compounds such as vanillin act as electron transport inhibitors (Moreland and Novitzky, 1987). Inhibition appears to be the result of alterations produced in the inner membrane by the allelochemical.

The concentration of compounds tested for inhibitory activity against cotton and wheat radicle elongation are listed in as milligrams per dish, corresponding to 2 ml of a 1 mM solution added to the dish (Table 4). The column of data for cotton shows that the two monoterpenes, *p*-cymen-9-ol and piperitenone, inhibited radicle elongation by 16 and 13%, respectively. The sesquiterpene alcohol, α-bisabolol, and vanillin were 9% and 11% inhibitory, respectively. All the compounds listed except vanillin were inhibitory to wheat radicles. The concentration of compounds used in this bioassay is relatively low and is equal to or below that used by other scientists (Colton and Einhellig, 1980; Patterson, 1981; Williams and Hoagland, 1982). Piperitenone, a monoterpene with an oxo group, was the most inhibitory compound tested. Asplund (1968) reported that monoterpene ketones such as camphor and pulegone were

TABLE 4. EFFECTS OF 1 mM CONCENTRATIONS OF AUTHENTIC VOLATILE COMPOUNDS ON COTTON AND WHEAT RADICLE ELONGATION

Compound	Plant part extracted	Concentration (mg/dish)	Inhibition[a] (%)	
			Cotton	Wheat
Control		0	0a	0a
α-Bisabolol	root	0.44	9bcd	39c
δ-Cadinene	root	0.41	2ab	20b
p-Cymen-9-ol	pod	0.30	13cd	34bc
Phenethyl alcohol	pod	0.24	6abc	28bc
Piperitenone	root	0.30	16d	43c
Vanillin	root, pod	0.30	11cd	2a

[a] Means followed by the same letter are not significantly different at the 5% level using a protected LSD.

the most inhibitory of all monoterpenes tested and were an order of magnitude greater in toxicity to radish seeds than HCN.

The "normal" essential oils of devil's-claw extracted from the upper plant parts on August 22 and 27 were inhibitory to cotton radicle elongation (Table 5). Piperitenone and α-bisabolol were included for comparison purposes. In this bioassay, 4 ml of the 1 mM essentiall oil solution was added to each dish; this corresponds to 0.80 mg/dish of essential oil. The essential oil was inhibitory to cotton radicle elongation to the same extent as the compounds piperitenone and α-bisabolol. Only the essential oil from August 14 inhibited wheat radicle, while piperitenone and α-bisabolol were extremely inhibitory. The essential oil contains up to 220 compounds, so that each compound is acting in only minute quantities.

Synergistic Effects. It is important to recognize the synergistic properties of components of essential oils that arew of different types and classes of compounds. These compounds individually may be phytotoxic, nontoxic, or stimulatory. An example is the inactive root essential oil isolated on September 10 (Table 2); it still contains α-bisabolol, δ-cadinene, piperitenone, and vanillin, which are allelochemical in the pure state. In the preliminary reports (Waller et al., 1987; Waller, 1989) on the allelopathic activity of devil's-claw normal essential oil, evidence was presented that vanillin, when added to the essential oil, produced an even more phytotoxic response. It shows that vanillin that occurs in the essential oil of the devil's-claw may control its allelochemical response. Similar situations may occur with other individual compounds of the essential oil; however, this is being investigated further at the present. Synergism has also been reported by Einhellig (1989), Fischer et al. (1989), and Tang et al. (1989), and it is well recognized that pronounced allelochemical activity occurs when different classes of compounds are present.

TABLE 5. EFFECTS OF 1 mM CONCENTRATIONS OF DEVIL'S-CLAW ESSENTIAL OILS FROM 1987 (BASED ON AVERAGE MOLECULAR WEIGHT OF 200) AND SELECTED COMPOUNDS ON COTTON AND WHEAT RADICLE ELONGATION

Sample	Concentration (mg/dish)	Inhibition[a] (%)	
		Cotton	Wheat
Control	0	0a	0a
Essential oil, 8/14	0.80	7ab	9b
Essential oil, 8/22	0.80	15bc	6ab
Essential oil, 8/27	0.80	12bc	4ab
Piperitenone (root)	0.60	17c	75d
α-Bisabolol (root)	0.89	12bc	42c

[a] Means followed by the same letter are not significantly different at the 5% level using LSD.

CONCLUSION

The essential oils collected from the upper portions of devil's-claw in late August were inhibitory to cotton, but those from the roots were not inhibitory. This is a very sensitive developmental stage for cotton in Oklahoma, involving the initiation of flowering and boll formation. Devil's-claw leaves and pods release volatile chemicals; this suggests that cotton growing nearby can be affected with subsequent yield reductions because of the toxicity of such chemicals.

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WINTER CHEMICAL DEFENSE OF ALASKAN BALSAM POPLAR AGAINST SNOWSHOE HARES

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Abstract—Palatabilities of parts and growth stages of balsam poplar (*Populus balsamifera*) to snowshoe hares (*Lepus americanus*) are related to concentrations of specific plant metabolites that act as antifeedants. Buds are defended from hares by cineol, benzyl alcohol, and (+)-α-bisabolol. Internodes are defended by 6-hydroxycylohexenone (6-HCH) and salicaldehyde. Although defense of internodes depends upon both compounds, the defense of juvenile internodes is principally related to salicaldehyde concentration; the defense of internode current annual growth is principally related to 6-HCH concentration. The concentration of 6-HCH can be supplemented by the hydrolysis of phenol glycosides when plant tissue is disrupted, raising the possibility of a dynamic element of the chemical defense of poplar.

Key Words—Balsam poplar, *Populus balsamifera*, snowshoe hare, *Lepus americanus*, plant chemical defense, herbivore, cineol, benzyl alcohol, bisabolol, 6-hydroxycyclohexenone, salicaldehyde.

INTRODUCTION

A variety of studies have demonstrated that during winter snowshoe hares (*Lepus americanus*) are highly selective in their feeding habits. Even though snow cover puts extreme limitations on the browse available to hares at this time, they often

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ignore large portions of the potential food supply. Field studies (Bookout, 1965; Keith et al., 1984; DeVos, 1964; Smith et al., 1988) indicate that hares utilize only certain species of woody plants and show dramatic preferences for parts and growth stages of the plants that they do consume. Cafeteria-style feeding trials have corroborated these observations at several different geographic locations (Bookout, 1965; Klein, 1977; Sinclair and Smith, 1984).

One of the most interesting plants from this perspective is balsam poplar (*Populus balsamifera*), a rapidly growing early successional tree that is often found in thick stands along riverbanks and in recently disturbed sites (Chapin et al., 1983; Van Cleve et al., 1983; Viereck and Little, 1972). Cafeteria feeding trials have shown that portions of the mature stage of poplar are moderately palatable to hares but that twigs of juvenile poplar are among the least palatable of all plant material offered (Sinclair and Smith, 1984; Klein, 1977). Futhermore, field observations indicate that even mature poplar is utilized very selectively by hares. Buds are ignored (Bryant and Kuropat, 1980; Sinclair and Smith, 1984), while small twigs are eaten and larger branches are extensively barked (DeVos, 1964).

Although the reasons for hares' selective use of poplar have never been determined, the suggestion has been made that it is a result of unpalatable secondary metabolites (Bryant and Kuropat, 1980; Sinclair and Smith, 1984). The study reported here was initiated to investigate three aspects of the potential relationship between plant chemistry and hares' discriminate use of poplar. Specifically, we investigated the relationship between balsam poplar chemistry and (1) the general unpalatability of poplar, (2) the hares' preference for mature over juvenile twigs, and (3) the hares' selective use of parts of mature poplar twigs.

METHODS AND MATERIALS

Extract Preparations

Crude extracts of poplar were prepared by extraction (10 liters of solvent per kilogram of plant material) of fresh, winter-dormant twigs (including buds) with freshly distilled diethyl ether (24 hr) followed by 50% aqueous methanol (12 hr) or by hot water extraction (10 min) of freeze-dried internodes. The ether extract was concentrated, dried ($MgSO_4$), filtered, and added to an amount of oatmeal equal to the fresh weight of poplar from which it was prepared. The treated oatmeal was placed in open pans in a fume hood for 24 hr to allow solvent to evaporate. Aqueous methanol and hot water extracts were concentrated on a rotary evaporator at 45°C and lyophilized. The resulting powder was sprinkled on oatmeal that had been slightly moistened with water. The treated oatmeal was stirred to distribute the extract evenly and then lyophilized.

A diethyl ether extract of internodes (stems between buds) was prepared and added to oatmeal as described above for the ether extract of intact twigs.

The diethyl ether-soluble portion of the steam distillate of poplar buds was prepared according to the procedure of Mattes et al. (1987) and added to oatmeal as described for the ether extracts. Subsequently, the steam distillate was separated by vacuum fractional distillation (Mattes et al., 1987). These fractions were individually added to oatmeal as described for the ether extracts of twigs, except distillation fractions were added to produce oatmeal that was 2% treatment by weight (approximately the concentration, on a fresh weight basis, of steam distillates in winter-dormant buds of mature poplar).

The final set of bioassay experiments utilized oatmeal treated with individual poplar metabolites (Mattes et al., 1987) found in pertinent crude fractions. Volatile liquids were added directly to oatmeal by pipet and were administered as uniformly as possible. Treated oatmeal was placed in a stoppered flask and allowed to stand (with occasional shaking) at room temperature for 24 hr before use in a feeding trial. Nonvolatile metabolites were evenly administered to oatmeal as ether solutions and solvent removed as described above for crude ether extracts.

Conduct of Bioassays

Pans of oatmeal treated with poplar extract or metabolite and pans with an equivalent amount of solvent-treated oatmeal (control) were offered in randomized pairs to captive hares. After 24 hr, we measured the amount of treated and control oatmeal the hares had eaten and used these values to calculate a preference index (mass of treated oatmeal eaten/mass of control oatmeal eaten). This index represents the degree to which poplar phytochemicals either deterred or stimulated hare feeding. Details of our bioassay protocol are described by Bryant (1981a), Reichardt et al. (1984), and Clausen et al. (1986). A paired t test was used to test for differences in the masses of control and treated oatmeal eaten.

Hare colonies in Alaska and Wisconsin each were established from individuals trapped locally. Thus the feeding trials with ether extract were conducted with a common protocol using animals from two extremes of the natural range of *L. americanus*.

Hare Preference for Poplar Growth Stages and Parts

Plant Samples. A set of feeding trials was conducted to quantify hare preference for growth stage and plant parts of balsam poplar. We selected six poplar populations separated by 10–125 km between Fairbanks and Nenana, Alaska. At each site, we collected four separate samples: (1) current annual growth (CAG) internodes of 1-year-old juvenile stage stump sprouts < 8 mm diameter,

(2) small (< 8 mm diameter) twigs from the 1- to 2-m-height zone of 4-year-old juvenile stage saplings containing both CAG and older internodes, (3) twigs < 8 mm diameter from the upper crown of 4- to 5-m-tall adult trees containing CAG and older internodes, and (4) 3-mm-thick strips of noncorky bark from larger upper crown branches of adult trees. Each sample contained material from 50 to 100 individual poplars. Twigs and bark from adult trees were out of reach of hares in most winters, whereas twigs of juvenile plants were from within the height range available to hares in winter. These collections of twigs and bark were used in feeding trials and in quantification of metabolites in poplar parts.

For six months prior to feeding trials, caged hares were offered similar twigs and bark from adult and juvenile poplar and commercial livestock chow (Quality Texture) ad libitum so neither adult nor juvenile poplar was a novel potential food at the time of the trial. The poplar used for acclimation came from a pooled sample from four of the six poplar populations we studied (those sites within 15 km of the University of Alaska). Before each feeding trial, we subsampled for chemical analysis buds, CAG internodes, older internodes, and noncorky bark from the poplar collection used in that trial. These subsamples were then subsampled to obtain (1) a fresh–dry mass ratio that was used to calculate the dry mass concentration of poplar phytochemicals in each part eaten by hares and (2) the percentage contribution of buds, CAG, and older internode biomass to the biomasses of twigs of both growth stages. This biomass distribution was used to estimate the mass of each part of each growth stage offered to hares and eaten by hares in our first feeding trial.

Feeding Trial 1. We compared hare preferences for six poplar populations and two growth stages (juvenile stump sprouts, mature trees) within populations. Nine individually cages hares were offered for 24 hr a randomized pair of 150-g fresh bundles of twigs from the upper crown of adult trees and stump sprouts from the same poplar population. The twigs from adult poplar contained CAG internodes, older internodes, and buds. The stump sprout twigs contained CAG internodes and buds. One such experiment was run daily for each of the six populations. Each hare had access to a limited amount of alternative food (20 g of Quality Texture Chow), so they would be forced to feed largely on poplar. After each 24-hr feeding trial, all internodes and buds remaining in each cage were collected, identified by growth stage, and weighed. The darker brown color of stump sprout CAG internodes as compared to adult twig CAG internodes allowed us to identify these two parts. The chalky grey color and, in the case of short shoots, the greatly reduced internode length allowed us to identify older internodes of adult poplar and differentiate them from adult poplar CAG internodes. The smaller size and more resinous appearance of stump sprout buds allowed us to separate with a reasonable degree of accuracy rejected buds of juvenile and adult poplars. However, this separation was not as reliable as was

separation of internodes of the two growth stages, so there may have been a slight error in our identification of buds. The fresh mass of each part eaten by each hare in each feeding trial was determined by subtraction of the mass of that part remaining after 24 hr from estimated mass of that part offered. We first analyzed the results of this experiment by nested ANOVA (SAS general linear models procedure) to estimate the relative contributions of populations and growth stages within populations to hare preferences for interior Alaskan balsam poplar in winter. We then analyzed the masses of poplar parts eaten (juvenile CAG internode, adult CAG internode, older internode, bud) by one-way ANOVA followed by a priori comparison of means (Sokal and Rohlf, 1969).

Feeding Trial 2. In a second set of six feeding trials, we measured the fresh masses of six poplar parts snowshoe hares ate in 24 hr when no alternative food was available. We offered to the same nine individually caged hares used in the first experiment 200 g of each poplar part (buds of adult plants, CAG from juvenile 4-year-old saplings, CAG from adult trees, older internodes from juvenile 4-year-old saplings, older internodes from adult trees, noncorky bark from larger branches of adult trees) for 24 hr. The poplar part used in each experiment came from a pooled sample obtained from all of the six poplar populations used in the first experiment. In the case of internodes, we offered hares adjacent 2-cm sections of CAG and older internode from the same twig to minimize effects of twig diameter upon feeding preferences. After 24 hr, we measured the total biomass of each twig part remaining in each hare's cage and subtracted that mass from the mass of that part offered. These experiments estimated the mass of each poplar part a hungry, but not starving, hare would eat per day. Data from these experiments were log $(x + 1)$-transformed prior to analysis by one-way ANOVA followed by a priori comparison of means. Additionally we used the Student-Newman-Keuls test ($\alpha = 0.05$) to test unplanned comparisons among pairs of means of part biomasses eaten.

Chemical Analysis

Secondary metabolites other than condensed tannins in poplar parts were quantitatively analyzed by chromatography (Mattes et al., 1987). Condensed tannin concentrations were determined by a modification of the proanthocyanidin method of Martin and Martin (1982), as described in detail in Bryant et al. (1985). The major sugars of winter-dormant twigs of woody plants (fructose, glucose, sucrose) (Kramer and Kozlowski, 1979) were extracted from ground lyophilized internodes (0.25 g) with water (2.00 ml at 90°C for 5 min). A 1.00-ml portion of extract was removed and added to 1.00 ml of water containing cellobiose (4.00 mg/ml) as an internal standard. This solution was filtered (0.45 μm) and analyzed by HPLC (amino column, 4.6 mm × 25 cm; CH_3CN–H_2O,

70:30, 1 ml/min). Peak detection was with a refractive index detector; retention times and response factors for fructose, sucrose, and glucose were determined with authentic samples of the sugars. The concentrations of mineral nutrients were determined colorimetrically by autoanalyzer (N, P) or by atomic absorption spectophotometry (Ca, Mg) as described by Reichardt et al. (1984). All chemical data were tested for skewness, kurtosis, and homogeneity of variances prior to analysis by one-way ANOVA (internodes) or paired *t* tests (buds). Because we had hypothesized before quantification that concentrations of certain metabolites in poplar internodes differed between growth stages and internode ages, a priori comparisons of means were used to test the statistical significance of these differences. We further tested the statistical significance of all unplanned comparisons of chemical differences among all pairs of poplar internodes by the Student-Newman-Keuls test ($\alpha = 0.05$).

RESULTS

Bioassays of Crude Extracts. The diethyl ether extract of intact stump sprouts (resin) deterred feeding by both Alaskan hares and Wisconsin hares (animals that represent populations from two extremes of the range of *L. americanus*), whereas the 50% aqueous methanol extract stimulated hare feeding and the hot water extract had a negligible effect on palatability (Table 1). Subsequent experiments with a diethyl ether extract of stump sprout internodes and

TABLE 1. EFFECTS OF CRUDE FRACTIONS FROM BALSAM POPLAR ON PALATABILITY OF OATMEAL TO HARES[a]

Part and fraction	Hares (*N*)	Preference index[b]	*P*
Twig			
Ether			
Alaska	18	0.02 ± 0.01	< 0.001
Wisconsin	5	0.01 ± 0.01	< 0.01
50% MeOH	9	3.28 ± 0.82	< 0.05
Hot water	9	1.63 ± 0.46	
Internode			
Ether	6	0.18 ± 0.13	< 0.01
Bud			
Steam distillate	5	0.00 ± 0.00	< 0.001

[a] Extracts were applied to oatmeal at the same concentrations (% dry mass) at which they occur naturally in juvenile poplar. Mean ± 1 SE.
[b] Preference index = mass treated oatmeal eaten/mass control oatmeal eaten.

the volatile (steam-distillable) portion of the ether-soluble metabolites of buds proved them both to be deterrent. Because the steam distillate of buds was the most deterrent of the fractions we tested (Table 1), we separated this fraction by vacuum fractional distillation into four subfractions, each of which was highly deterrent to hares (Figure 1).

Bioassays of Pure Compounds. After we identified most of the major components of the ether extract of internodes and the ether-soluble portion of the steam distillate of buds (Mattes et al., 1987), we tested the deterrence of each identified component in separate experiments (Table 2). Because we did not determine the absolute configurations of several of the compounds, in some cases we submitted both enantiomers (separately) or racemates for bioassay.

Three of the major bud metabolites (cineol, benzyl alcohol, (+)-α-bisabolol) were strong feeding deterrents. The remaining bud metabolites tested had no significant effect upon preference (Table 2).

Among internode metabolites, salicaldehyde and 6-hydroxycyclohexenone (6-HCH) were deterrent at 1% dry mass and benzyl gentisate was deterrent at 4% dry mass. Because of the difficulty in obtaining enough pure (+)-6-HCH from poplar to conduct bioassays, we also bioassayed (racemic) synthetic 6-HCH (Mattes et al., 1987) and found it to be about as unpalatable as the natural enantiomer (Table 2).

The high deterrence of ether extracts of internodes and buds and the steam distillate of buds (Table 1) compared to deterrence of subfractions of the bud steam distillate (Figure 1) or individual metabolites (Table 2) suggests either:

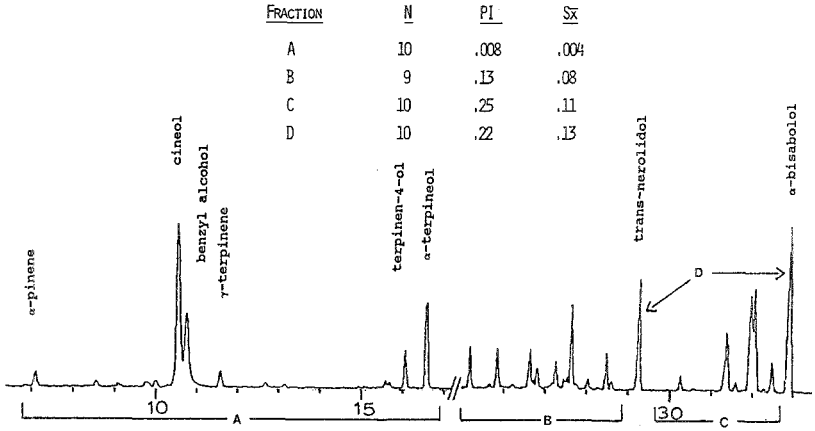

FIG. 1. Gas chromatographic trace (units of horizontal axis = min) of balsam poplar bud steam distillate, identities of major components, and bioassay results from subfractions of the steam distillate. Mean preference index (PI) ± 1 SE presented for subractions at 2% concentration.

TABLE 2. EFFECTS OF PURIFIED COMPOUNDS FROM BALSAM POPLAR AND THEIR MIXTURES ON PALATABILITY OF OATMEAL TO SNOWSHOE HARES

Compounds	Concentration (% dry mass)	Hares	Preference index	P
Bud secondary metabolites				
Benzyl alcohol	1.0	8	0.29 ± 0.10	0.001
Cineol	1.0	8	0.11 ± 0.07	0.001
(+)-α-Bisabolol	1.0	7	0.20 ± 0.06	0.001
(−)-α-Bisabolol	1.0	7	0.20 ± 0.08	0.001
(+)-α-Pinene	1.0	8	2.60 ± 1.10	NS
(−)-α-Pinene	1.0	8	9.50 ± 5.17	NS
α-Terpinene	1.0	8	2.39 ± 0.91	NS
γ-Terpinene	1.0	8	8.61 ± 3.36	NS
(±)-α-Terpineol	1.0	7	0.52 ± 0.20	NS
(+)-α-Terpinen-4-ol	1.0	7	0.59 ± 0.24	NS
(−)-α-Terpinen-4-ol	1.0	7	0.89 ± 0.40	NS
Internode secondary metabolites[b]				
(+)-6-HCH	1.0	8	0.44 ± 0.11	0.01
(±)-6-HCH	1.0	7	0.31 ± 0.08	0.01
Salicaldehyde	1.0	6	0.31 ± 0.09	0.01
Cyclohexane-1,2-dione	1.0	6	2.15 ± 1.02	NS
Benzyl gentisate	4.0	6	0.03 ± 0.02	0.01
Mixture A		8	0.15 ± 0.07	0.001
Mixure B		6	1.10 ± 0.38	NS
Sugars				
Fructose	10.0	5	3257 ± 417	0.05
Glucose	10.0	5	2.14 ± 0.72	NS
Sucrose	10.0	5	1275 ± 638	0.05

[a] Preference index = (Mass Treated Oatmeal Eaten/Mass Control Oatmeal Eaten).
[b] Mixture A contained 6-HCH and salicaldehyde at 1.4% and 0.6% respectively (the concentrations found in 1-year-old stump sprout internodes). Mixture B contained 6-HCH and salicaldehyde at 0.7% and 0.1% respectively (the concentrations found in noncorky bark of branches from crowns of mature balsam poplar). Both mixtures contained cycohexane-1,2-dione at 0.25% concentration.

(1) a synergistic component to the deterrent effects of these metabolites when they are present in the mixtures found in the plant or (2) significant deterrent effects associated with some of the unidentified metabolites (e.g., components of fraction B, Figure 1). Nevertheless, the results of these bioassay experiments clearly identify the important contributions of cineol, benzyl alcohol, and (+)-α-bisabolol to the unpalatability of poplar buds and suggest that levels of 6-HCH and salicaldehyde in the internodes may affect their palatabilities significantly.

Because of the feeding stimulation associated with the aqueous methanol extract (Table 1), we bioassayed the major sugars found in woody plants (fruc-

tose, glucose, sucrose) (Kramer and Kozlowski, 1979). Fructose and sucrose significantly stimulated feeding, and glucose tended to stimulate feeding (Table 2).

Plant Parts. When offered a choice of poplar stump sprouts or twigs from adult plants (Figure 2), hares fed almost entirely upon older internodes of adult plants, ate little CAG of either growth stage and no buds. ANOVA indicated differences in palatability among populations and growth stages ($F = 125$; $P < 0.0001$). However, most of the experimental variance (87%) was explained by growth stage palatability differences. A priori comparisons indicated stump sprout CAG internodes are less palatable than internodes of adult poplar ($P < 0.0001$) and that within adult twigs CAG internodes are less palatable than older internodes ($P < 0.0001$).

When hares were offered individual parts of poplar (Figure 3), buds were not eaten, CAG internodes of both growth stages and older internodes of 4-year-old juvenile-stage saplings little eaten, and older internodes and noncorky branch bark from crowns of adult poplars preferred. Thus, hare preferences for parts of winter-dormant Alaskan balsam poplar are buds < current year internodes of juvenile plants < older internodes of juvenile plants = current year internodes of mature plants < older internodes of adult plants < noncorky bark from larger branches from the crowns of adult plants. One-way ANOVA followed by a priori comparison of means (Table 3) indicated that these differences are statistically significant ($P < 0.0001$).

Relationships between Hare Preferences and Chemistry. Having demonstrated the presence of palatability-reducing metabolites in buds and current-year internodes of juvenile balsam poplar (stump sprouts), we measured the

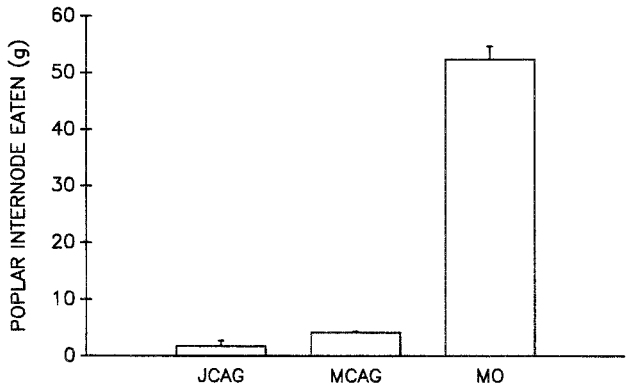

FIG. 2. Palatability of balsam poplar growth stages and parts when bundles of intact twigs were offered to hares. Mean ± 1 SE presented. Abbreviations are: JCAG = juvenile current annual growth, MCAG = mature current annual growth, MO = mature older growth.

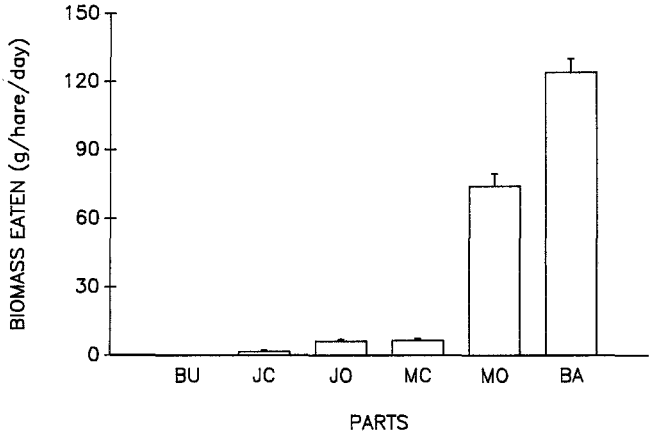

FIG. 3. Fresh masses of poplar parts eaten by snowshoe hares in 24 hr. Mean ± 1 SE presented. Abbreviations are: BU = bud, JC = juvenile current annual growth, JO = juvenile older growth, MC = mature current annual growth, MO = mature older growth, BA = bark.

levels of these substances (along with several others) in relevant plant material (Table 4).

The buds of juvenile-phase poplar (stump sprouts mixed with 4-year-old saplings) had higher concentrations of two major feeding deterrents (cineol and benzyl alcohol) than did buds of adult poplar. However, the levels of these compounds [plus (+)-α-bisabolol] in buds of both juvenile and adult poplar were similar and high enough to explain the unpalatability of all poplar buds to hares.

TABLE 3. ANOVA FOR BALSAM POPLAR PARTS FEEDING TRIAL

Source of Variation	SS	*df*	MS	*F*	*P*
Biomass parts eaten	30.76	5	6.15	307.60	0.0001
Bud vs. internode/bark	10.80	1	10.80	540.00	0.0001
Bark vs. internode	8.96	1	8.96	448.00	0.0001
Internode					
Juvenile vs. mature	5.08	1	5.08	254.00	0.001
Within juvenile	1.18	1	1.18	59.00	0.001
Within mature	4.74	1	4.74	237.00	0.001
Error	1.07	48	0.02		
Total	31.83	53			

[a]The biomass parts eaten sum of squares decomposed into planned comparisons among means by the a priori orthogonal comparisons method (Sokal and Rohlf, 1969). Data log $(x + 1)$-transformed (Zar 1974).

TABLE 4. CONCENTRATIONS OF SELECTED FEEDING DETERRENT SECONDARY METABOLITES, MINERAL NUTRIENTS, AND SUGARS IN PARTS OF WINTER-DORMANT BALSAM POPLAR FROM SIX POPLAR POPULATIONS LOCATED NEAR FAIRBANKS, ALASKA (MEAN ± 1 SE).

	Buds		Current internode		Older internode		
Constituents (% dry mass of plants)	Juvenile plants	Mature plants	Juvenile plants	Mature plants	Juvenile plants	Mature plants	Bark mature
Secondary metabolites							
Benzyl alcohol	0.29 ± 0.02^a	0.19 ± 0.03^b					
Cineol	0.49 ± 0.05^a	0.27 ± 0.05^b					
Bisabolol	2.65 ± 0.31^a	2.39 ± 0.43^a					
6-HCH			1.39 ± 0.11^a	1.39 ± 0.16^a	1.20 ± 0.07^a	0.94 ± 0.18^b	0.68 ± 0.11^b
Salicaldehyde			0.60 ± 0.12^a	0.17 ± 0.05^b	0.38 ± 0.11^a	0.12 ± 0.03^b	0.08 ± 0.04^b
Condensed tannin	1.48 ± 0.09^a	1.50 ± 0.11^a	0.00 ± 0.00^b	0.00 ± 0.00^b	0.01 ± 0.01^b	0.00 ± 0.00^b	0.04 ± 0.03^b
Mineral nutrients							
Nitrogen	1.91 ± 0.08^a	1.64 ± 0.07^b	1.41 ± 0.03^c	1.18 ± 0.06^d	0.95 ± 0.02^e	0.87 ± 0.05^e	0.77 ± 0.05^e
Phosphorus	0.27 ± 0.01^a	0.22 ± 0.01^b	0.19 ± 0.01^c	0.15 ± 0.01^d	0.13 ± 0.01^d	0.10 ± 0.01^e	0.09 ± 0.01^e
Calcium	4.94 ± 0.46^a	6.12 ± 0.55^a	8.12 ± 0.46^b	9.11 ± 0.25^b	8.18 ± 0.31^b	9.88 ± 0.25^c	10.95 ± 0.26^c
Magnesium	2.52 ± 0.17^a	2.46 ± 0.10^a	1.57 ± 0.10^b	2.63 ± 0.21^a	1.19 ± 0.17^b	1.73 ± 0.13^b	1.71 ± 0.18^b
Sugars							
Fructose			1.13 ± 0.12^a	0.82 ± 0.06^a		0.75 ± 0.06^a	
Glucose			1.07 ± 0.04^a	0.70 ± 0.04^b		0.59 ± 0.04^b	
Sucrose			0.87 ± 0.09^a	1.35 ± 0.13^a		1.07 ± 0.08^a	

[a] Values of a given compound with different superscripts differ at the $P < 0.05$ level of probability. The statistical significance of difference used for all comparisons among means were tested by the Student-Newman-Keuls test with the exception of bud secondary metabolites where a paired t test was used.

CAG internodes of juvenile poplar had a higher concentration of salicaldehyde than did CAG of mature internodes but about the same 6-HCH concentration (Table 4). Within both juvenile and mature growth stages, CAG internodes had slightly higher (but statistically insignificant) concentrations of both substances than older internodes. However, the unpalatable CAG of poplar twigs (Figure 3) contained twice as much total feeding deterrent as preferred older internodes of adult poplar ($P < 0.001$, paired t test). The combined mass of salicaldehyde and 6-HCH in winter-dormant balsam poplar internodes is juvenile CAG internodes > juvenile older internode = adult CAG internodes > adult older internodes, precisely the inverse to hare preferences for poplar internodes (Figures 2 and 3). Thus, it seems likely that the snowshoe hares' selective use of balsam poplar internodes in winter is controlled by salicaldehyde and 6-HCH. This conclusion is supported by the high preference for bark (Figure 3) and the low concentrations of both substances in balsam poplar bark (Table 4). The final test of this hypothesis was a pair of bioassay experiments with combinations of 6-HCH, salicaldehyde, and 1,2-cyclohexandione (a minor internode metabolite) (Mattes et al. 1987) at levels equivalent to those found in juvenile-stage current-year internodes (least palatable internode) and mature bark (most palatable poplar part). The results (10-fold preference for the mixture simulating bark; Tables 2 and 4) were consistent with the proposal that these compounds strongly influence the hares' selective use of older poplar internodes and bark.

We next investigated the role of benzyl gentisate, a less volatile internode metabolite, in poplar internode palatability. Bioassay experiments revealed that it was extremely deterrent to hares at 4% concentration (Table 2), a concentration below those found in internodes that were eaten by hares (Table 4). The solution to this apparent paradox was revealed when we discovered that benzyl gentisate is an artifact produced enzymatically (Figure 4) (Mattes et al., 1987) from its glucoside, trichocarpin (Pearl and Darling, 1969, 1971), during extraction and that no free benzyl gentisate can be detected when the transformation is circumvented during extraction. We have not bioassayed purified trichocarpin since neither the aqueous extract nor the aqueous methanol extract of poplar twigs (both of which contain substantial quantities of trichocarpin) deter hare feeding (Table 1). Thus, trichocarpin apparently has no significant effect on the palatability of poplar internodes (or, at best, has a small effect that is overriden by the stimulatory effects of sugars), but it can be viewed as a latent source of a deterrent (benzyl gentisate).

During our work with the trichocarpin–benzyl gentisate interconversion, we discovered another enzymatic transformation, that may contribute to the general unpalatability of balsam poplar internodes. A second major balsam poplar glucoside, salicortin (ca. 7% of the dry weight mass of poplar internodes as determined by isolation), can be converted by an enzyme preparation from

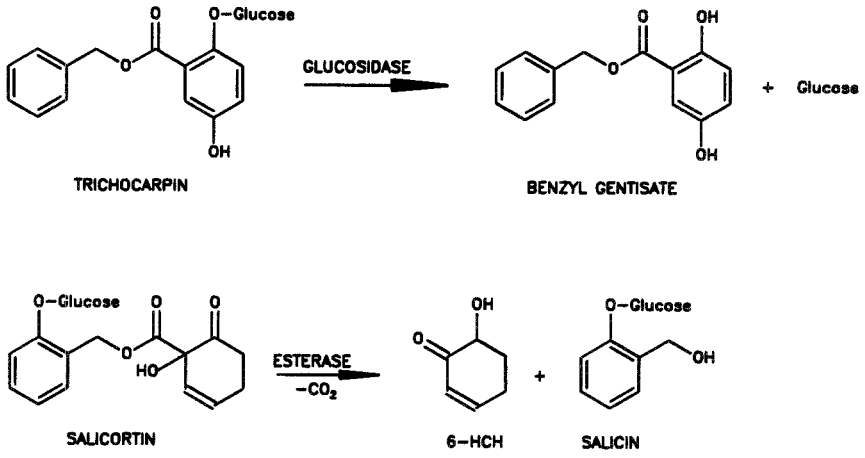

FIG. 4. Biochemical transformations of poplar metabolites with potential significance in defense against hares.

poplar internodes to 6-HCH (Figure 4) (Mattes et al., 1987). Thus, the static levels of 6-HCH in winter-dormant internodes may be augmented by hydrolysis of salicortin to 6-HCH as the plant is crushed during feeding.

In contrast to the relationships between palatabilities of plant parts and the concentrations of unpalatable secondary metabolites in those parts, we found no apparent relationships between the concentrations of mineral nutrients, sugars, or tannins and hare preferences for winter-dormant balsam poplar growth stages or parts. Relative concentrations of major mineral nutrients and sugars in plant parts generally decline as palatability of the plant tissue increases (Figures 2 and 3; Table 4). Of the four mineral nutrients we measured (N, P, Ca, Mg), only Ca concentrations seemed to correlate roughly with palatability of poplar parts. Because the unpalatable buds were the only plant part found to contain tannins (Table 4), these substances clearly cannot explain the large differences in palatability among age classes of internodes and bark (Figures 2 and 3).

DISCUSSION

Chemistry and Poplar Palatability. Feeding experiments in Alaska (Klein, 1977; Bryant, 1981a,b) and the adjacent Yukon Territory of Canada (Sinclair and Smith, 1984) have established that, during winter, snowshoe hares feed preferentially upon adult (Kozlowski, 1971) balsam poplar internodes as compared to similar-diameter internodes of juvenile-phase stump sprouts. When adult individuals are windthrown or cut down, upper crown branches are com-

pletely barked, whereas smaller twigs are not browsed as heavily, indicating a preference for noncorky bark over internodes and buds. Hares in both regions have strong preferences for certain parts within both growth stages. Internodes <8 mm diameter and noncorky bark of larger branches are eaten, and resinous buds are rejected (Bryant and Kuropat, 1980; Sinclair and Smith, 1984).

All parts and growth stages of poplar contain unpalatable secondary metabolites. As previously suggested (Bryant and Kuropat, 1980; Sinclair and Smith, 1984), bud resin defends this part from hare predation in winter, but its defensive properties are due to several—but not all—of its components. Thus, even in a mixture as complex as a plant resin, biological properties are dependent upon individual metabolites and/or synergisms involving individual metabolites (Reichardt et al., 1987). Internodes also contain unpalatable chemicals. In particular, salicaldehyde appears to be the primary defensive metabolite of the juvenile-phase poplar we studied, whereas 6-HCH appears to defend CAG internodes of both stages (Table 5). However, defense of juvenile poplar may involve a synergistic effect of both substances or effects of other metabolites not isolated and evaluated in this study. Indeed, Jogia et al. (1989) have recently reported that 2,4,6-trihydroxychalcone, a metabolite of balsam poplar from the Yukon Territory, acts as an antifeedant for hares.

Biochemical transformations that occur during herbivory may also influence internode palatability. Two major metabolites found in poplar internodes, trichocarpin and salicortin (ca. 8% and 7% on a dry mass basis, respectively), do not affect palatability directly but are converted to unpalatable metabolites

TABLE 5. ANOVA FOR SECONDARY CHEMISTRY OF INTERNODES OF TWIGS USED IN FEEDING EXPERIMENT COMPARING PALATABILITY OF WHOLE TWIGS FROM JUVENILE OR MATURE BALSAM POPLAR[a]

Source of variation	SS	*df*	MS	*F*	*P*
Internode chemistry	9.65	5	1.93	32.16	<0.0001
6-HCH vs. salicaldehyde	7.46	1	7.46	124.33	< 0.0001
Juvenile vs. mature					
6-HCH	0.20	1	0.20	3.33	NS
Salicaldehyde	0.84	1	0.84	14.00	< 0.001
Within mature					
6-HCH	0.59	1	0.59	9.83	< 0.01
Salicaldehyde	0.56	1	0.56	9.33	< 0.01
Error	1.90	30	0.06		
Total	11.55	35			

[a] The internode sum of squares decomposed into planned comparisons among means by the a priori orthogonal comparison method (Sokal and Rohlf, 1969).

(benzyl gentisate and 6-HCH, respectively) upon disruption of plant tissue. Thus, there may be a dynamic element to poplar's defense of internodes (i.e., one that becomes active only when the plant is eaten) similar to the defense provided by cyanogenic glycosides, which become toxic to the plant or animal only when hydrolyzed during feeding (Jones, 1972).

In assessing the relative contributions of "static" and "dynamic" elements to the palatability of poplar internodes, it is important to recognize that hare predation on poplar is of significance only in winter (Wolff, 1980). During this period, low ambient temperatures reduce the rates of the two pertinent hydrolysis reactions in the plants (relative to reaction velocities observed under laboratory conditions) according to normal kinetic principles. In addition, the lower temperatures limit the extent of hydrolysis reactions by greatly reducing the plants' supply of liquid water (through desiccation and/or freezing of plant-contained water) needed for hydrolysis. Thus, while providing an interesting example of a complex dynamic defense, the hydrolytic reactions of trichocarpin and salicortin within the plant are probably not of great practical significance to defense of poplar internodes in winter. Once these compounds enter the animal, however, our laboratory experience indicates that conditions are adequate to promote rapid hydrolysis to a toxic substance, which may promote postingestive distress that results in a learned aversion (Garcia et al., 1985).

Chemical Limitations on Balsam Poplar Consumption by Hares. Our results identify the chemical "signals" that influence hares' selective use of poplar, but, as discussed by Reichardt et al. (1987), they do not provide the physiological reasons that snowshoe hares avoid poplar buds and juvenile internodes. The physiological basis for this avoidance is suggested by other studies. Antimicrobial activities have been ascribed to the major bud metabolites cineol (Agarwal and Mathela, 1979), benzyl alcohol (Karabit et al., 1986), and especially α-bisabolol (Dull et al., 1957; Szalonkai et al., 1976). While the internode metabolite trichocarpin does not affect palatability and may itself be rather innocuous physiologically, its hydrolysis by β-glucosidases presumably present in the hares' digestive system (Nisizawa and Hashimoto, 1970) would release benzyl gentisate, to which strong antimicrobial properties have been ascribed (Klöpping and van der Kerk, 1951). Ingestion of such a substance could be very detrimental to lagomorphs such as hares that depend heavily upon microbial degradation of plant tissues and microbial production of vitamins for their nutrition (Cheeke, 1987). A similar, but more complex, scenario can be envisaged for salicortin. Ester hydrolysis—catalyzed by ingested plant esterase, digestive enzymes, or low pH—would release 6-HCH. At the very low pH (1–2) encountered in the stomach of lagomorphs (rabbits; Cheeke, 1987), 6-HCH is chemically converted to phenol (Mattes et al., 1987), a demonstrated toxin to mammals (Sax, 1979; Villagines, 1982) and an antimicrobial agent (Leifertova et al., 1975). Although less likely because of the anerobic conditions, 6-HCH

might be converted to catechol in the cecum (Clausen et al., 1989). Catechol is a demonstrated antimicrobial agent (Leifertova et al., 1975) and an inhibitor of vitamin B synthesis (Reese, 1979). Both the antimicrobial properties of catechol and blocking of vitamin B synthesis would be detrimental to hares. Furthermore, unmetabolized 6-HCH could directly upset the hare's metabolism and physiology because it is an electrophile (Clausen et al., 1989) capable of bonding to a variety of biological bases (e.g., basic amino acid side chains in proteins). In short, ingestion of any poplar parts by a hare would introduce a variety of toxins and antimicrobial agents into its digestive system. While the hare can probably tolerate moderate quantities of such substances, ingestion of large amounts of poplar buds or juvenile internodes would probably cause digestive malfunction because of major changes in populations of digestive microbes, directly poison the hare, reduce absorption of amino acids, and/or block vitamin B synthesis.

Neither low protein (Sinclair et al., 1982; 1988b) nor inhibition of protein digestion by tannins (Sinclair and Smith, 1984) seems to be a chemical limitation to use of Alaskan balsam poplar internodes and bark by snowshoe hares. We found hare use of balsam poplar parts was inversely related to the nitrogen concentrations of these parts. Thus, while protein content is clearly an important factor in determining food quality, hares do not select food solely on the basis of total nitrogen (protein) content. Our results also demonstrate that even though hares show strong preference for certain parts of internodes (Figures 2 and 3), none of these parts contain detectable levels of condensed tannins (Table 4). Interestingly, Sinclair and Smith (1984) report high levels of protein-binding phenolics in balsam poplar from the nearby Yukon Territory. If these protein-binding phenolics are condensed tannins, balsam poplar in Alaska and the adjacent Yukon have quite different biochemistries, in contrast to the low variation we found among Alaskan balsam poplar populations.

Even though there appear to be some significant differences in the reasons for snowshoe hares' selective use of balsam poplar in different locations, it is becoming clear that specific metabolites of poplar are responsible for its defense. Sinclair and Smith (1984) have previously examined three general hypotheses for food choices of hares and have concluded that none of them adequately explain hares' selection of food. They suggested that a viable alternative explanation was that plant defense is based on specific phytochemicals, a proposal consistent with the results of a variety of studies of snowshoe hare–woody plant interactions (Reichardt et al., 1984, 1987; Clausen et al., 1986; Sinclair et al., 1988a; Jogia et al., 1989). The results presented here and those of Jogia et al. (1989) suggest that, indeed, hares' discriminant use of poplar—both with respect to growth stage and parts—is directly related to concentrations of specific plant metabolites.

CONCLUSIONS

We traced the unpalatability of balsam poplar parts to snowshoe hares to a set of specific secondary metabolites. Buds, the least palatable part of the winter-dormant plant, are protected by at least three components of a complex mixture of volatile compounds, principally mono- and sesquiterpenes. Internodes are chemically defended by two volatile compounds, possibly aided by two glucosides which probably convert to toxic substances following ingestion. The hares' relative preferences for bark of upper crown branches of adult plants > older internodes of adult plants > CAG internodes of adult plants = older juvenile plants > CAG internodes of juvenile plants are probably governed by levels of these compounds, although some uncertainty remains because of the chemical complexity of internodes and the importance of potential chemical transformations of poplar internode metabolites following ingestion.

Defense of Alaskan balsam poplar internodes against winter browsing by snowshoe hares can be viewed as having two components. One, related to the physiological age of the plant, is based upon salicaldehyde. The other, related to the chronological age of internodes, is largely based upon 6-HCH in the case of adult plants and the combined effects of 6-HCH and salicaldehyde in the case of juvenile plants. In both cases, CAG internodes are more heavily defended chemically than older internodes or bark.

Taken together, these conclusions point out that an understanding of plant–herbivore interactions requires an appreciation of (1) the identity of individual chemicals involved, (2) the chemical conversions that occur during herbivory, and (3) the control woody plants have over allocation of defenses with respect to age and phenophase.

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GERMACRONE DEFENDS LABRADOR TEA FROM BROWSING BY SNOWSHOE HARES

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Abstract—Labrador tea (*Ledum groenlandicum*), a slow-growing late successional evergreen, is highly unpalatable to snowshoe hares (*Lepus americanus*). Germacrone, a sesquiterpene that is the major component of the essential oil of *L. groenlandicum*, was shown by bioassay to be a potent antifeedant to hares. Its concentrations in leaves and internodes of the plant are high enough to defend *L. groenlandicum* from hares. This chemical defense of Labrador tea from herbivory is consistent with the resource availability theory of antiherbivore defense.

Key Words—Labrador tea, *Ledum groenlandicum*, snowshoe hare, *Lepus americanus*, herbivore, plant chemical defense, germacrone.

INTRODUCTION

Labrador tea is a slowly growing evergreen shrub found on infertile soils in late successional evergreen forests, bogs, muskegs, and heathlands throughout boreal portions of the world (Chapin, 1980; Hulten, 1968; Viereck and Little, 1972). In North America there are two generally recognized species, *Ledum palustre*

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ssp. *decumbens* [Ait.] Hulten and *Ledum groenlandicum* Oeder, although morphological distinction is sometimes problematic (Welsh, 1974).

One distinctive ecological feature of North American Labrador tea is that neither species is utilized to any significant extent by herbivores. Studies in Arctic Alaska have shown that, of tundra shrubs studied, the leaves of *L. palustre* were least preferred by lemmings (Batzli and Jung, 1980), ground squirrels (Batzli and Sobaski, 1980), voles (Batzli and Jung, 1980), caribou (Kuropat, 1984), reindeer (Trudell and White, 1981), and four species of lepidopteran larvae (MacLean and Jensen, 1985). *L. groenlandicum* has reported to be virtually untouched by herbivores in Quebec (Prudhomme, 1983) and Ontario (Reader, 1979), although a population explosion of the measuring worm in 1978 resulted in severe defoliation of *L. groenlandicum* in an Ontario bog (Reader, 1979); Keith et al. (1984) reported this species is not eaten by snowshoe hares during winter at a site in Alberta.

According to the resource availability theory of plant defensive evolution (Bryant et al., 1983; Coley et al., 1985), slowly growing evergreens such as Labrador tea should be effectively defended against herbivores. Indeed, it is known that both these species of Labrador tea contain large quantities of essential oils and that their chemical compositions differ markedly (Von Schantz and Hiltunen, 1971), but the defensive properties of these metabolites have not been tested.

The study reported here was designed to determine if *L. groenlandicum* has the predicted (Bryant et al., 1983; Coley et al., 1985) chemical defenses and, if so, to define the defensive chemicals.

METHODS AND MATERIALS

Feeding Trials with Twigs. To verify the field observation of Keith et al. (1984) that Labrador tea has very low palatability for snowshoe hares, we conducted a five-day midwinter feeding trial that compared the palatability of Labrador tea with six other common boreal woody species. Four of the species were mature growth form (Kozlowski, 1971), comparatively fast-growing deciduous shrubs. Two of them (*Salix alaxensis* and *S. bebbiana*) are preferred winter foods of snowshoe hares, and two (*Betula glandulosa* and *S. arbusculoides*) are occasionally used by hares. The other two species, juvenile white spruce (*Picea glauca*) and juvenile black spruce (*P. mariana*) are slowly growing evergreens (Viereck and Little, 1972; Chapin, 1980; Chapin et al., 1983) that are little used as food by snowshoe hares (Klein, 1977; Bryant et al., 1983, 1985; Keith et al., 1984; Sinclair and Smith, 1984; Sinclair et al., 1988). Twigs <4 mm in diameter were collected from the height range used by snowshoe hares in winter, 0–0.5 m above the snow. Ten captive snowshoe hares were

maintained on a mixture of these twigs (100 g of fresh twigs of each species offered each day) supplemented with 30 g of rabbit chow (Quality Texture) for two weeks prior to the feeding trial. On the first day of the trial, each hare was offered pans containing 400 g of fresh twigs of each of the seven species as 2 cm segments. On each subsequent day, the species most preferred on the previous day was removed, so the hares were forced to feed on a diet of decreasing palatability as the trial progressed. Throughout the trial, the total twig biomass offered each hare each day (2800 g) was held constant and equally divided among the species offered. Thus, on day 5, the last day of the trial, the hares were offered 933 g of each of the three least-preferred species. No chow supplement was provided during the feeding trial. The amount of each species eaten each day was obtained by subtraction (biomass offered − biomass remaining). This type of feeding trial, which has been used previously by Bookhout (1965) in studies of snowshoe hare feeding behavior, provides two indices of hare preferences for woody browse, an estimate of the biomass of each species eaten on day 1 and the maximum biomass of each species eaten on one day of the feeding trial.

Preparation of Extracts. Fresh branches (leaves and stems) of winter-dormant *L. groenlandicum* were soaked for 24 hr in distilled diethyl ether (1 liter ether/100 g plant material). After filtration of plant material, the ether solution was dried ($MgSO_4$), filtered, and concentrated under vacuum. The filtered plant material was subsequently extracted with distilled methanol (24 hr; 1 liter methanol/100 g plant material), and the filtered solution was concentrated under vacuum.

Fresh plant material was also subjected to continuous steam distillation and the distillate isolated as described by Reichardt et al. (1989).

Identification of Germacrone. Germacrone was isolated from the steam distillate and identified as described in Reichardt et al. (1989).

Identification of Ursolic Acid. Concentration of the dried ether extract from 100 g of *L. groenlandicum* to about 50 ml produced a precipitate (0.37 g). Recrystalization of the solid from ether gave 0.21 g of a white solid (mp = 224–228°C) which was converted to its methyl ester (CH_2N_2/ether), subsequently identified as methyl ursolate from its [^{1}H]- and [^{13}C]NMR spectra (Romeo et al., 1977; Seo et al., 1975).

Extract Bioassays. Ether extracts were applied as $MgSO_4$-dried ether solutions to oatmeal in an open pan, and the treatment was stored in a fume hood overnight to allow the solvent to evaporate. The methanol extract was applied as a methanol solution and the solvent removed under vacuum. The steam distillate and germacrone were applied as solutions in ethyl acetate, and the solvent was evaporated as with the ether solutions. Pans of treated oatmeal and solvent-treated oatmeal control were offered in a randomized array to captive hares. After 24 hr, weights of remaining samples were recorded and the amount of

each sample eaten was calculated. Additional details of our bioassay procedure are described elsewhere (e.g., Reichardt et al., 1984; Clausen et al., 1986).

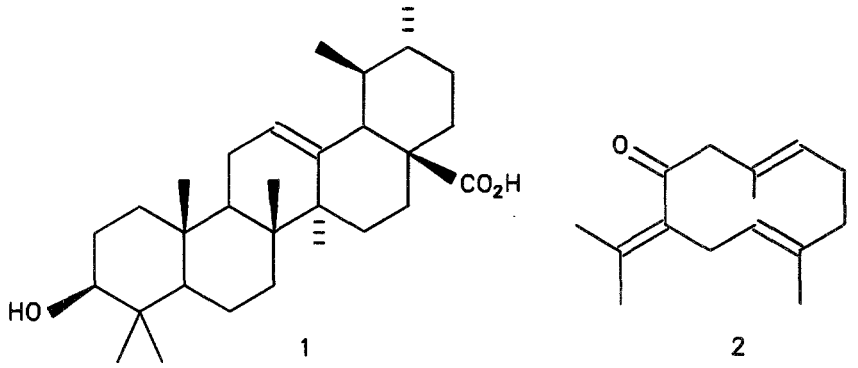
Quantification of Germacrone in Plant Tissue. Twigs of *L. groenlandicum* were collected from about 50 individual plants at each of six sites near Fairbanks, Alaska, during midwinter. Leaves and internodes were separated by hand and the internodes cut at the scar between current-year growth (CAG) and older-than-current-year growth (OG). Pooled samples (ca. 0.2 g) of leaves, CAG, and OG were cut into small pieces and soaked for 24 hr in 6.0 ml dichloromethane containing 1.33 mg camphor (internal standard). Filtered extracts were analyzed by gas chromatography [Perkin Elmer 8410 gas chromatograph equipped with a flame ionization detector and a 30-m × 0.53-mm RSL-200 (Alltech) column; He flow = 6 ml/min; injector and detector temperatures = 300°C; temperature program was 100°C for 2 min, 100–180°C at 10°C/min, 180°C for 4 min, 180–230°C at 10°C/min]. Germacrone concentrations in each sample were calculated using the Perkin Elmer data station and a carefully determined (Reichardt et al., 1989) response factor for germacrone relative to camphor.

Statistical Analysis of Data. The results of feeding trials with twigs, bioassays of crude extracts and germacrone, and assays of the concentrations of germacrone in *L. groenlandicum* parts (leaves, CAG internodes, older internodes) were log $(x + 1)$ transformed prior to analysis by parametric statistics, because all data sets contained comparatively large values and values close to or equal to zero (Zar, 1974). Data from feeding trials with twigs and assays of germacrone concentrations in *L. groenlandicum* parts were analyzed by one-way ANOVA followed by a priori comparison of means (Sokal and Rohlf, 1969). Data from bioassays were analyzed by the paired t test.

RESULTS

Feeding Trials with Twigs. On the first day, hares did not eat any evergreen browse (Figure 1). On day 5, when only the three evergreen species were offered, Labrador tea was eaten less than the two spruces ($P < 0.0001$). The maximum biomasses of the deciduous species eaten exceeded that of the evergreens ($P < 0.0001$), and the maximum biomasses of the two spruces exceeded that of Labrador tea ($P < 0.0001$). Furthermore, the hares did not eat leaves or current-year internodes of Labrador tea even when they did eat small amounts of older growth of this plant.

Identification of Major Metabolites of L. groenlandicum. Thin-layer chromatography of the ether extract of *L. groenlandicum* indicated two major components. One of these substances precipitated as the extract was concentrated and, after conversion to its methyl ester, was identified as ursolic acid (**1**) (Scheme 1). Germacrone (**2**), the other major metabolite, constituted about half



SCHEME 1.

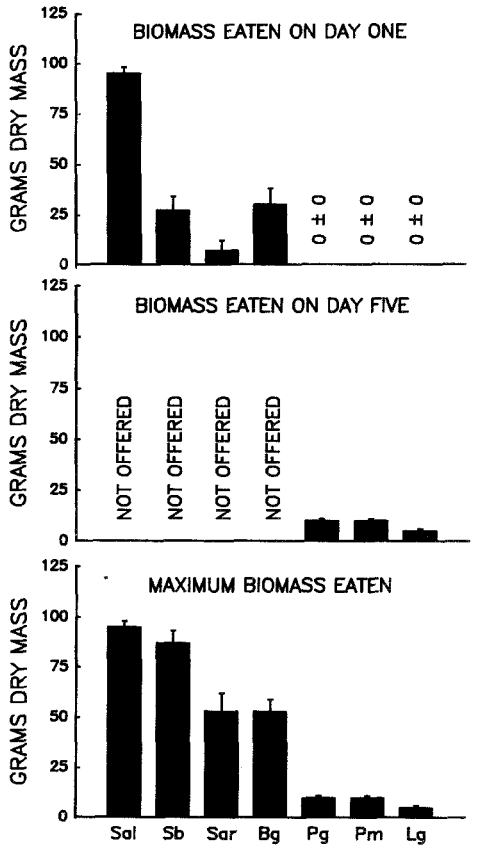


FIG. 1. Biomasses of twigs of seven common Alaskan woody species eaten by snowshoe hares. Sal = *S. alaxensis*, Sb = *S. bebbiana*, Sar = *S. arbusculoides*, Bg = *B. glandulosa*, Pg = *P. glauca*, Pm = *P. mariana*, Lg = *L. groenlandicum*, Mean ± 1 SE presented.

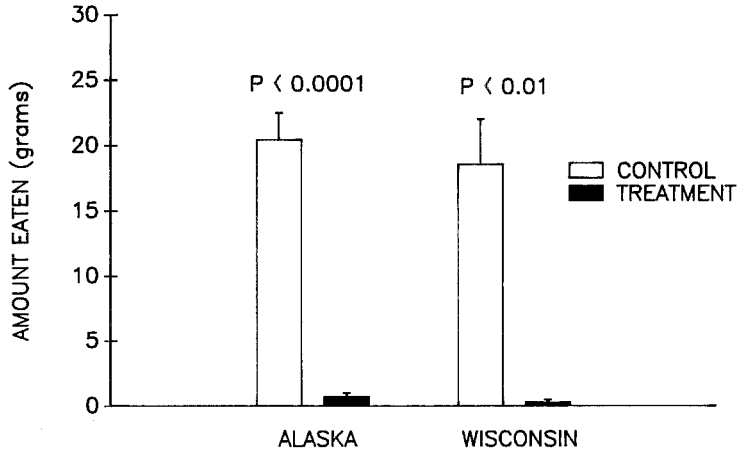


FIG. 2. Amount of untreated oatmeal and oatmeal treated with the crude diethyl ether extract of *L. groenlandicum* eaten by snowshoe hares in Alaska and Wisconsin. Mean ± 1 SE presented.

of the essential oil (Reichardt et al., 1989), as anticipated from earlier reports (Von Schantz and Hiltunen, 1971).

Bioassays of Crude Fractions. Initial experiments in Alaska and Wisconsin demonstrated that the ether-soluble metabolites of *L. groenlandicum* deter hare feeding (Figure 2). A subsequent experiment (Figure 3) demonstrated that the

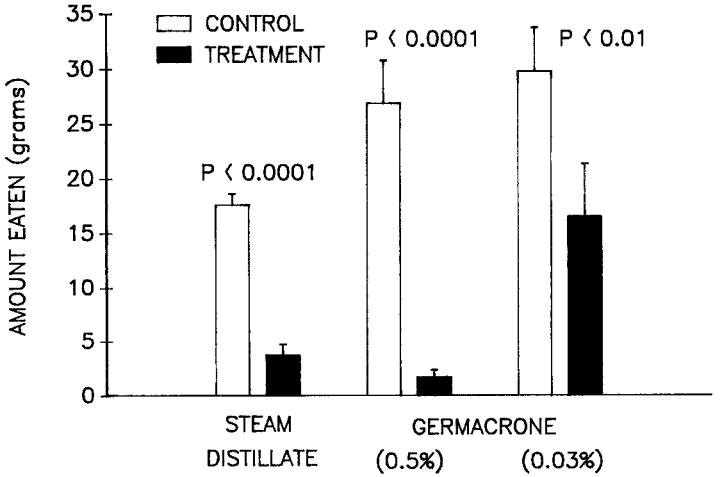


FIG. 3. Amount of untreated oatmeal and oatmeal treated with the steam distillate of *L. groenlandicum* and germacrone eaten by snowshoe hares in Alaska and Wisconsin. Mean ± 1 SE presented.

TABLE 1. CONCENTRATIONS OF GERMACRONE IN LEAVES AND INTERNODES OF *L. geoenlandicum*[a]

Plant part	Germacrone (%)	
	$\bar{X}$	SE
Leaves	0.53	0.06
Current growth internodes	0.028	0.006
Older internodes	0.014	0.004

[a] Means and standard errors for % germacrone (dry mass basis) are given for plants pooled from collections at each of six sites and analyzed in triplicate.

steam distillate of this plant (a subfraction of the ether extract) also deters hare feeding. Addition of the methanol-soluble, ether-insoluble fraction to oatmeal had no significant effect on palatability to hares.

Bioassays of Germacrone. Oatmeal impregnated with germacrone at concentrations approximating those found in leaves (0.5%) and current-year internodes (0.03%) of *L. groenlandicum* deterred hare feeding (Figure 3).

Quantification of Germacrone in Plant Tissue. The concentrations of germacrone in leaves, current-growth internodes, and older internodes of plants collected at six sites in interior Alaska were found to be 0.5%, 0.03%, and 0.01%, respectively (dry weight basis), by gas chromatographic analysis (Table 1).

DISCUSSION

Results from feeding trials (Figure 1) and field observations (Keith et al., 1984) indicate that Labrador tea is highly unpalatable to snowshoe hares. Our bioassays of crude extracts and purified germacrone demonstrate that the unpa-

TABLE 2. ANOVA OF GERMACRONE CONCENTRATIONS IN LEAVES AND INTERNODES OF *L. groenlandicum*

Source of variation	SS	*df*	MS	*F*	*P*
Parts	47.04	2	23.52	52.72	0.0001
Leaf vs. internodes	43.77	1	43.77	115.69	0.0001
Between internodes	3.27	1	3.27	8.64	0.0148
Error	5.68	15			
Total	52.72				

latability of *L. groenlandicum* to snowshoe hares can be explained by germacrone. Germacrone certainly provides an adequate defense of leaves, and even the low levels of germacrone found in internodes probably defend these parts from hares. Possible defensive effects of other metabolites [e.g., tannins (Reader, 1979) and ursolic acid] are likely to supplement the germacrone-based defense.

The relationships among the distribution of germacrone within the plant (Tables 1 and 2), the hares' discriminant use of plant parts in our feeding trial, and the hares' use of *L. groenlandicum* parts in the field are noteworthy. Of particular interest is the fact that the foliage of *L. groenlandicum*, which is strongly defended by germacrone, is not used as food by Alaskan snowshoe hares in winter (Bryant, unpublished). It is even less palatable than the highly unpalatable foliage of juvenile spruces (Klein, 1977; Bryant et al., 1983; Sinclair and Smith, 1984; Sinclair et al., 1988), which is the only other major source of green foliage available to Alaskan snowshoe hares in winter. The elevated concentration of germacrone in current-year growth (CAG) over its concentration in older-than-current-year growth (OG) follows the pattern seen in other woody plants (e.g., Provenza and Malechek, 1983; Chapin et al., 1985; Yabaan et al., 1985; Meyer and Karazov, 1989). Although the defensive significance of the difference in germacrone concentration in CAG and OG has not been assessed, it is likely that this difference accounts for the hares' discrimination between CAG and OG.

In several respects, *L. groenlandicum* is an ideal plant for testing the hypothesis that slowly growing, late successional plants are strongly defended against herbivores (Bryant et al., 1983; Coley et al., 1985). Not only does *L. groenlandicum* fit the growth and habitat patterns predicted for highly defended plants, but it also is less palatable than juvenile white spruce and juvenile black spruce, which are also slow-growing evergreens (Bryant et al., 1983; Chapin et al., 1983). Moreover, *L. groenlandicum* is less apparent (sensu Feeny, 1976) than juvenile white spruce and juvenile black spruce, because it is covered by snow for much of the winter except at the base of spruce where the lower branches protect it from burial by snow. Thus, the low palatability of *L. groenlandicum* and our demonstration that a carbon-based secondary metabolite, germacrone, can account for its low palatability provide strong support for the resource availability theory of plant antiherbivore defense (Bryant et al. 1983; Coley et al. 1985).

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UPWIND SEARCHING FOR AN ODOR PLUME IS SOMETIMES OPTIMAL

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Abstract—A model is presented that suggests that the optimal strategy for an animal walking or crawling on a substrate while searching for the source of a chemical carried by a shifting wind or current may be to move upwind (or against the current). The requirements are that (1) the current direction fluctuates rapidly within a range that exceeds 30° on both sides of the mean direction, and (2) the searching animal can move accurately up- or downwind and, once within the influence of the chemical plume, can move efficiently to the source. Under these conditions, an upwind search is shorter (on the average) by 0.9–0.3 of the range of influence of the chemical stimulus. Thus, this strategy is relatively more important when the total search path is short, i.e., when sources are typically close by. The mean length of the downwind search path is given by

$$\langle L_D \rangle = d + r[(1/E) + \cos \alpha]/2$$

and the upwind search path by

$$\langle L_U \rangle = d + r[(2/E) - (\alpha/\sin \alpha) - \cos \alpha]/2$$

where d is the starting distance up- or downwind of the source, r is the range of stimulus influence, E is the efficiency with which the searcher moves to the source within the influence of the plume, α is the angle of the maximum extent of wind direction from the mean, and the average, $\langle\ \rangle$, is taken over all starting positions across the wind direction that lead to search paths that intercept the area swept by the plume.

Key Words—Search, plume, odor, wind, current.

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INTRODUCTION

Although there has been a great deal of research on the behavior of insects in an odor plume, little attention has been paid to behavior leading to discovery of the plume (Cardé, 1981; Sabelis and Schippers, 1984). It has often been said that the optimal strategy is to move in a straight line perpendicular to the wind direction (e.g., Linsenmair, 1973; Cardé, 1981, 1984; Janzen, 1984). Recent analysis has quantified the presumed advantage of searching in straight paths (Pline and Dusenbery, 1987; Dusenbery, 1989b). However, animals have been observed to search in directions other than crosswind (Kalmus, 1942; Linsenmair, 1969; Finch and Skinner, 1982), and recent analyses suggest circumstances in which other directions are superior.

In the case of a flying animal, the optimal direction is more or less downwind, depending on how elongated the plume is (Dusenbery, 1989d). This is because the animal can move downwind faster with the same effort. In contrast, an animal moving on a substrate generally requires the same effort to move in any direction. In this circumstance, crosswind searching is more likely to be optimal.

However, Sabelis and Schippers (1984) have demonstrated that if the wind direction shifts sufficiently rapidly over a range of $\pm 30°$ or more from the mean direction, it is more effective to search parallel to the wind than across it. This is because the plume sweeps out an area that has a greater cross section across the wind than along it. They further argue that downwind searching is more effective than upwind despite presenting a contrary biological example. I will demonstrate that a more complete analysis of this situation suggests that upwind search may be more effective, as has been observed in a variety of insects. Although the discussion will refer to wind, the arguments apply equally well to plumes formed by currents of water. A related analysis has analyzed the advantage of pulsed pheromone release in improving the efficiency of chemical communication (Dusenbery, 1989c).

METHODS AND MATERIALS

The model presented here is based on the same assumptions as used by Sabelis and Schippers (1984). It assumes that the mean wind direction is constant but that the instantaneous wind direction fluctuates rapidly within a range of angles, $\pm\alpha$, from the mean wind direction. The instantaneous plume of effective length, r, thus sweeps out a sector of radius r subtending an angle 2α. The fluctuations are rapid compared to the speed of movement of the searching animal, so it encounters the instantaneous plume as soon as it enters the swept area. It is further assumed that the searching animal can determine the mean wind direction and move accurately with or against it.

RESULTS

The analysis presented by Sabelis and Schippers (1984) is limited by the fact that they considered only the cost of reaching the plume and not the total cost of reaching the source. In order to evaluate the total cost, consider the path length the searcher must travel when starting a distance d up- or downwind of the source and a distance y across the wind from the source. The situation is diagrammed in Figure 1, assuming the searcher can move straight to the source once it is within the area swept by the plume. For a downwind search, the path length traveled to reach the source is

$$L_D = d + \left(\frac{1 + \cos \alpha}{\sin \alpha}\right) y$$

For an upwind search it is

$$L_U = d + r - (r^2 - y^2)^{1/2} y$$

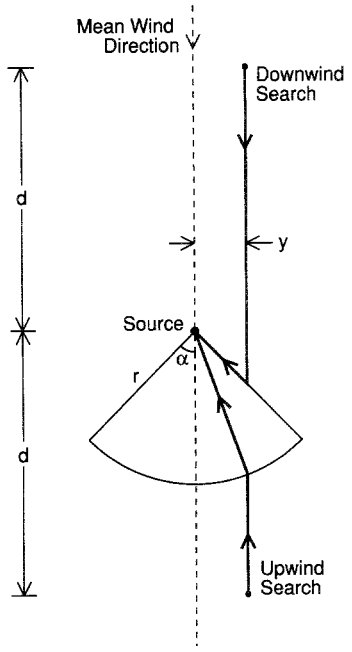

FIG. 1. Geometry of the search paths. The sector-shaped area represents the plume. The paths of an upwind and a downwind search are illustrated by heavy lines. Both searches start an equal distance from the source in directions parallel (d) and perpendicular (y) to the mean wind direction. In this example, $\alpha = 45°$ and y is the mean value of all starting points that intercept the swept area.

The mean path lengths for all values of y that intercept the plume are

$$\langle L_D \rangle = d + \frac{r}{2}(1 + \cos \alpha)$$

and

$$\langle L_U \rangle = d + \frac{r}{2}\left(2 - \frac{\alpha}{\sin \alpha} - \cos \alpha\right)$$

with $r < d$. Both averages have the form $\langle L_i \rangle = d + rc_i(\alpha)$, where $c_i(\alpha)$ is a function of α only. For α ranging from 0 to 90°, $c_D(\alpha)$ changes monotonically from 1 to 0.5 and $c_U(\alpha)$ from 0 to 0.215 (Figure 2, solid lines). Consequently, the upwind path is shorter for all values of α. The difference in path length ranges from r (for small α) to about 0.3 r (for large α). We are primarily interested in α between 30° and 90°, since for smaller α, crosswind searching is optimal (Sabelis and Schippers, 1984). At 30°, the values have not changed much from $\alpha = 0°$: $c_D(30°) = 0.93$ and $c_U(30°) = 0.04$. And the difference in path length is $0.89r$.

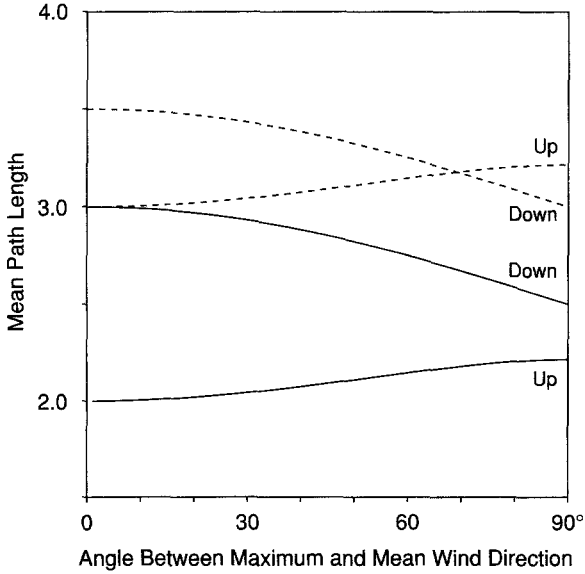

FIG. 2. Search path lengths. The mean path length for various values of α are plotted for starting distances twice the stimulus range from the source ($d = 2r$). The path lengths are given as a proportion of the stimulus range (L/r). The solid lines are for an efficiency of orientation within the sector of stimulus influence of 100% ($E = 1.0$). The dotted lines are for 50% efficiency ($E = 0.5$). For each set of conditions, the mean path of both up- and downwind searches are presented, as indicated.

The assumption that an animal in the area swept by the instantaneous plume can move straight toward the source can be relaxed. The efficiency of orientation (E) within the sector swept by the plume may be defined as the ratio of the length of the shortest possible path to the mean actual path length (Dusenbery, 1989a). If the efficiency is not 100%, then on average $\langle L_U \rangle$ is increased by $r[(1/E) - 1]$ and $\langle L_D \rangle$ by half as much. The relations become

$$\langle L_D \rangle = d + \frac{r}{2}\left(\frac{1}{E} + \cos \alpha\right)$$

$$\langle L_U \rangle = d + \frac{r}{2}\left(\frac{2}{E} - \frac{\alpha}{\sin \alpha} - \cos \alpha\right)$$

Such inefficiency decreases the advantage of upwind search. Up- and downwind paths are equal when $1/E = 2 \cos \alpha + (\alpha/\sin \alpha)$. For $\alpha = 30°$, $E \approx 0.36$ produces equal mean path lengths. For $\alpha = 90°$, equal paths occur with $E = 2/\pi \approx 0.64$. For smaller values of E, downwind searching is more efficient. The case of $E = 0.5$ is depicted in Figure 2.

DISCUSSION

This analysis clearly demonstrates that upwind searching can be most efficient if (1) wind variability is sufficiently high to give the effective plume a broader cross section perpendicular to the wind than parallel to it, and (2) once in the area swept by the plume, the searching animal can move efficiently to the source. The relative advantage of upwind (vs. downwind) searching is greater for searches of short total path length. Thus, this strategy is more important when targets are expected to occur at high density and the likely path is not too much greater than the range at which the plume can be detected. In this situation, the advantage of upwind searching can exceed 50%.

It should be emphasized that this model is appropriate to animals walking or crawling on a substrate but generally not to flying animals, because it is assumed that locomotion is slow compared to movement of the plume and that costs are equal for movement in any direction.

Unfortunately, there are few cases where appropriate data for testing the predictions of this model are available. Walking fruit flies (Kalmus, 1942) and dung beetles (Linsenmair, 1969) have been observed to move upwind in the absence of known chemical stimuli; the model presented here provides a possible explanation of these observations. Upwind movement of the cabbage root fly, *Delia radicum*, has been observed (Finch and Skinner, 1982). Although root flies move by flying fast in short hops, they apparently orient to the wind while on the ground. Thus, the model presented here could apply to their behavior.

It is hoped that this paper will inspire researchers to investigate the param-

eters in this model with a variety of animals. It might well apply to slugs, in addition to walking insects. For a thorough test of the model, it would be desirable to obtain information on (1) wind variability in the natural environment in order to estimate α, (2) the range of attraction to estimate r, (3) the typical density of sources to estimate average d values, and (4) the efficiency with which the searching animals move toward the source, once within the area swept by the plume.

When this analysis suggesting upwind search is combined with those suggesting downwind search (Sabelis and Schippers, 1984) and crosswind search (Dusenbery, 1989d), it may be concluded that the optimal search direction is a complex function of environmental conditions, and one may question whether many animals would adopt a strategy of searching in a particular direction. The answer probably depends on how predictable a particular environment is. Some flying insects have been observed to search in all directions (Elkington and Cardé, 1983) in a forest environment in which wind direction is extremely variable (Elkington et al., 1987).

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ROLES OF AMINO ACIDS, PROTEIN, AND FIBER IN LEAF-FEEDING RESISTANCE OF CORN TO THE FALL ARMYWORM

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Abstract—The free amino acids have been shown by isolational work and choice bioassays to be more important than all other factors evaluated in defining leaf-feeding resistance of corn (*Zea mays* L.) to fall armyworm (FAW) [(*Spodoptera frugiperda* J.E. Smith)] larvae. 6-MBOA (6-methoxy-benzoxazolinone) and maysin, toxins present in corn, were shown not to be significant factors for leaf-feeding resistance to first-instar FAW larvae because of their low concentrations in the whorl. Amino acid analysis showed that while the ratios of the essential amino acids in susceptible (S) and resistant (R) lines were similar, there were differences in the nonessential amino acids, particularly aspartic acid, which was higher in R lines. Also, the ratio of essential amino acids to nonessential amino acids was important, being too low in expressed whorl leaf juice (obtained from V_8–V_{10} growth stage plants) to support larval growth, although juice was stimulatory in choice tests. The total protein content of whorls in S lines was about 15% higher than in R lines, but the significance of this difference is uncertain, because nutritional tests showed that larval growth increased with total protein only up to 12% protein. Sugars were only slightly stimulatory. Thus, the amino acids along with higher hemicellulose content of R lines, established by us earlier, appear to explain much of the basis of resistance in corn to larval leaf-feeding of the FAW.

Key Words—Fall armyworm, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, corn, *Zea mays*, plant–insect interaction, amino acids, herbivory, feeding resistance.

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INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), (FAW) is a major pest of corn, *Zea mays* L., in the southeastern United States (Davis et al., 1988a,b). The damage to corn is caused by larvae feeding primarily on leaves, but also on husks and ears. The damage caused by this insect is extensive; in fact, late-planted corn can be destroyed.

A long-continuing corn breeding program at Mississippi State, Mississippi, has led to the release of eight germplasm lines with leaf-feeding resistance to the FAW and the southwestern corn borer (SWCB), *Diatraea grandiosella* Dyar (Davis et al., 1988a,b). Additionally, these lines have shown resistance to the sugarcane borer, *Diatraea saccharalis* (Fab.), the corn earworm, *Heliothis zea* (Boddie), and the European corn borer (ECB), *Ostrinia nubilalis* (Hubner) (Davis et al., 1988b). The resistance appears to be operating by the same mechanisms (antibiosis and nonpreference), so there is the expectation that the same or similar chemical and physical factors may govern the resistance of corn to these insects (Wiseman et al., 1981, 1983; Williams et al., 1985, 1987; Davis et al., 1988a,b).

Previous chemical work showed that lines resistant to both the SWCB and FAW were consistently higher in crude fiber, and the hemicellulose content of the fiber was higher while the cellulose content was unchanged. The susceptible lines were at least 25% higher in protein, lipid, total sugars, ash, and polyphenol oxidase activity (Hedin et al., 1984).

6-Methoxybenzoxazolinone was reported as a plant resistance factor to the ECB (Klun and Brindley, 1966), but we have found it to be only one third as toxic to the FAW and SWCB as to the ECB in larval feeding studies (Nicollier et al., 1982) and present in very low concentrations in corn lines developed in Mississippi with leaf-feeding resistance to the SWCB and FAW (Hedin et al., 1984). Maysin, a flavone glycoside, was isolated from corn silks of the line Zapalote Chico by Waiss et al. (1979), who reported it to have antibiotic activity toward the corn earworm *Heliothis zea* (Boddie). However, we were not able to find maysin in whorl tissue, the site of usual FAW feeding (Hedin et al., 1984).

This report describes the chemical differences between leaf-feeding resistant and susceptible corn genotypes relative to these insects and evaluates these constituents for their capability to promote or deter FAW larval feeding and growth.

METHODS AND MATERIALS

Corn Lines and Harvesting. Corn lines used in these studies were developed by the Corn Host Plant Resistance Research Unit at Mississippi State, Mississippi. They were selected for desirable resistance properties to the SWCB

and FAW and grown using normal agronomic practices in the area. Whorls were excised on site from plants in the midwhorl V_{8-10} (Ritchie and Hanway, 1982) stage of growth, transported to the laboratory where they were frozen at −20°C, and dissected to obtain the inner green–yellow tissue that constitutes the feeding site. This section was later freeze-dried, ground in a Wiley mill, and the powder stored in a freezer until analyzed. Whorl juice was harvested as described in a later section.

FAW Larval Growth on Corn Plants in the Field. Growing plants were infested in the field using the procedures of Davis et al. (1988a,b). After 10 days, the plants were dissected and the larvae recovered, counted, and weighed. FAW larval weights on the hybrids included in this study were previously reported by Williams et al. (1989) and are listed here for background information: (S) Ab24E × SC229, 63.33 mg; Ab24E × Tx601, 68.76 mg; Ab24E × Va35, 41.31 mg; SC229 × Tx601, 59.83 mg; SC229 × Va35, 48.88 mg; Tx601 × Va35, 39.13 mg. S average = 53.54 mg. (R) Mp496 × Mp704, 24.76 mg; Mp496 × Mp706, 28.72 mg; Mp496 × Mp78:518, 26.50 mg; Mp704 × Mp78:518, 32.55 mg; Mp706 × Mp78:518, 23.56 mg. R average = 22.68 mg.

Processing and Chromatography of Cold-Pressed Corn Whorl Juice. Upon pressing corn whorls containing about 85% moisture in a laboratory press at 10,000 psi, about 45–50% could be recovered as a juice which yielded 2.5–4.0% total solids. After filtration of the juice to remove a small amount of remaining particulate material, 200-ml portions of the juice were chromatographed on a 5 × 70-cm Sephadex G-50-150 column with water as the eluant. Eight to ten 250-ml fractions were collected, of which 80–85% of the total solids eluted as a brown band, mostly sugars and amino acids, at about 1200–1500 ml. Quantitative recovery of total solids from the Sephadex G-50-150 column was demonstrated by weighing the fractions after freeze-drying.

The sugars were separated from the amino acids by chromatography on a 2 × 6-cm Dowex 50 column that was prepared by equilibration with HCl, followed by washing with water. The sample (1–2 g) was dissolved in water and introduced onto the column. The sugars were recovered by further elution with water until reaction of aliquots with anthrone demonstrated that all sugars had been eluted. The amino acids were subsequently eluted with 10% NH_4OH until reaction of aliquots with ninhydrin demonstrated that all amino acids had been eluted. There was a quantitative recovery (as determined by weighing of the freeze-dried eluates) of total solids in the two fractions; 75% of the total solids in the neutral fraction were mono- or disaccharides, and 92% of the total solids in the NH_4OH fraction were amino acids.

Analytical Procedures. Association of Official Analytical Chemists (AOAC) methods (Horwitz, 1975) were used for the following analyses: total solids (moisture), 14.083; crude fat, 14.019; crude fiber, 14.118; acid detergent fiber 7.055, 7.056, 7.057; ash, 14.114; total protein, 2.049 (percent nitrogen × 6.25); nitrogen-free extract (NFE), by difference from 100%. Total sugars

were determined by the colorimetric anthrone procedure (DuBois et al., 1956). Protein also was determined colorimetrically by the Coomassie blue test (Bradford, 1976). All results are reported on a dry weight basis.

Amino acids were determined by HPLC analyses before and/or after acid hydrolysis, employing their phenylthiocarbamyl derivatives (Cohen et al., 1986). Individual sugars were also determined by HPLC analysis; employing a Waters Bondapak NH_4 column and acetonitrile–water (80:20).

Isolation and Analysis of Maysin. Silks were removed from young ears of Zapalote Chico, Stowell's Evergreen, SC229 × Tx601, and Mp496 × Mp701. The silks were ground in 70% aqueous acetone, and the contents were filtered, concentrated under vacuum, and analyzed by HPLC employing a C_{18} reverse-phase column. The elution regime was 90% aq. H_3PO_4–10% acetonitrile, 10 min; then a gradient of 70% aq. H_3PO_4–30% acetonitrile to 100% acetonitrile, 20 min. Authentic maysin samples provided by Dr. Maurice Snook, Athens, Georgia, and Dr. Anthony Waiss, Albany, California, were used as analytical standards.

FAW Choice Laboratory Bioassays. Bioassays were conducted using extracts or known constituents applied in water solution to filter paper disks (2.5 cm) placed in Petri dishes, and the larvae were allowed to express preference according to the procedure of Williams et al. (1987). Results are reported as the average number of larvae responding to the test minus those responding to the water check, or in confrontational tests, test A minus test B, unless otherwise described.

FAW Larval Growth Feeding Tests. Larval growth tests were conducted according to the rearing procedures established by Davis (1989) with modifications described elsewhere in this text. The ingredients of the standard casein–wheat germ diet (used for test comparisons) and the test diet (without the protein sources casein and wheat germ) were custom-prepared by Bio-Serv, Inc., Frenchtown, New Jersey.) The pH of the diet was adjusted to 5.65.

Two dietary tests were conducted with freeze-dried whorls. In the first, 11 g of the ground whorls were suspended in 250 ml of a 2% agar mixture, warmed, and then allowed to gel in 30-ml cups. FAW larvae were allowed to feed for 14 days. In the second test, 10, 20, or 30 g of whorl powder and 20, 10, or 0 g of alphacel in 150 ml H_2O were added to 300 ml of the standard casein–wheat germ diet. Larvae were weighed after seven and 10 days.

Statistical Methods. Data were statistically evaluated by analysis of variance or by calculation of standard errors of the means or of standard deviation.

The statistical design used to compare larval growth on diets based on the analysis of amino acids from several sources was completely random. Each treatment was generally comprised of 40 plastic cups (30 ml) using one larva per cup. In some tests where dehydrated whorl leaf tissue was evaluated, a randomized complete block design was used with eight replications. Each treatment was six cups, each containing one larva.

RESULTS AND DISCUSSION

Preference Studies

Feeding Stimulation of FAW in Choice Bioassays of Whorl Juice. In three preliminary tests, an average of 7.0 larvae were attracted to juices from two susceptible (S) lines, while an average of 3.0 were attracted to juices from two resistant (R) lines. In a second test, a portion of the S and R juices were freeze-dried and reconstituted to the original water volume, or reconstituted and dialyzed, then both fractions reconstituted to the original water volume. The insects responded more strongly (average responding to test minus average responding to water check) to the S × S juice (6.4) than to the R × R juice (2.4). Freeze-drying did not appear to decrease the response, and the dialysis test showed that the stimulatory factors were more concentrated in the dialyzable (low-molecular-weight) fraction. In a third test with whorl juices in which the insects gave greater responses, the results were: S × S = 16.2 ± 1.4, R × R = 12.0 ± 1.6, and S × S vs. R × R = 11.2 ± 1.7. Thus, FAW larvae were stimulated to feed by both S × S and R × R juices, but preferred the S × S in a confrontational test with R × R juices.

Fractionation and Choice Bioassay Testing of Whorl Juices. Susceptible and resistant whorl juices were fractionated on a Sephadex G-50-150 column employing water as eluant. Most of the total solids (average of 85%) were eluted from the column as a brown band from about 1200 to 1500 ml. Various diagnostic tests showed that sugars (average of 69%) and amino acids (13%) were the predominant components of the brown band. About 4% of the total solids were accounted for by proteins and polysaccharides, and an additional 8% that eluted later than the brown band as minerals, inositol, and phytic acid. The bioassay results indicated that all of the fractions were somewhat active (range of averages of test minus check = 3.5–7.8 and the brown band = 6.8). Because the brown band comprised the greatest portion of the total solids (85%), it appeared to be most responsible for the stimulation of feeding. None of the juice fractions was deterrent. Therefore, no evidence could be found for the presence of an antifeedant.

Analysis and Bioassay of Whorl Juice Sugars and Amino Acids. Analysis of the brown-band sugars by HPLC showed that two, glucose and fructose, were present in the ratio of approximately 2 : 1. No other mono- or disaccharides were evident in the chromatogram. The brown-band amino acids were analyzed by the HPLC procedure of Cohen et al. (1986), and the results are presented in Table 1.

To determine which of these two fractions most stimulated feeding of FAW larvae, freeze dried isolates were reconstituted at 50 mg/ml so that 3 drops (0.17 ml) contained 7 mg of solids, the same quantity in 3 drops of whorl juice containing 4% total solids. The results (summarized in Table 2) show that the

TABLE 1. AMINO ACIDS IN CORN WHORL JUICE, FREEZE-DRIED WHORL, CORN CALLUS TISSUE, KERNELS, CASEIN, AND WHEAT GERM[a]

	Total amino acids (%)									
			Whorl juice		Freeze-dried whorl		Callus		Kernels	
Amino acid	Casein	Wheat germ	S	R	S	R	S	R	S	R
Arginine	3.7	11.6	1.0	1.0	5.5	5.5	4.0	3.5	5.0	5.4
Histidine	2.8	2.5	0.7	1.6	1.5	1.6	1.4	1.3	2.3	2.3
Isoleucine	5.4	3.9	0.8	1.0	4.4	4.8	3.6	3.4	4.7	4.5
Leucine	8.2	6.8	1.0	1.0	7.9	8.9	6.3	5.9	15.3	15.0
Lysine	7.3	7.7	1.3	0.8	7.4	7.2	5.5	5.6	4.2	3.7
Methionine	2.5	0.6	0.3	0.2	2.4	2.3	0.8	1.0	0.5	1.2
Phenylalanine	4.5	3.8	0.7	1.6	5.3	5.0	4.2	4.2	6.5	6.5
Threonine	4.4	4.4	1.8	2.8	4.2	3.6	3.2	2.8	3.2	2.8
Tryptophan[b]	1.5	(1.5)	(1.5)	(1.5)	(1.5)	(1.5)	(1.5)	(1.5)	—	—
Valine	6.5	5.4	2.1	1.5	5.3	5.3	4.7	4.3	5.0	5.5
Alanine	2.7	6.9	18.9	21.3	9.7	9.8	9.3	9.4	8.3	7.7
β-Alanine			0.8	1.0						
γ-Aminobutyric acid			4.1	2.3						
Asparagine[c]			25.3	21.7	10.5	7.9	7.6	10.3		
Aspartic acid[c]	6.4	10.3	5.1	6.8	3.5	5.5	10.1	17.2	5.8	6.3
Creatinine			5.1	1.4						
Cysteic acid		0.5			1.7	1.6	1.7	1.6		
Cystine	0.3	0.4					1.0	0.4	0.3	0.6
Glutamic acid	20.0	14.8	2.9	4.0	9.8	10.0	14.9	13.7	19.9	19.5
Glycine	2.5	6.3	8.3	9.5	5.9	6.2	4.8	4.5	3.1	3.3
Phosphoserine			0.2	0.6						
Proline	10.1	4.8	5.5	2.1	5.6	4.8	18.5	15.2	10.0	9.4
Serine	5.7	4.6	16.4	17.2	7.2	7.2	4.3	4.1	4.1	4.2
Tyrosine	5.7	2.8	1.0	2.1	2.5	2.9	1.6	2.0	2.2	2.3
Essential amino acids (%)	46.8	48.6	11.2	13.0	45.4	45.7	35.2	33.5	46.7	46.9
Amino acids in sample (%)	88.0	31.0	11.5	13.0	18.4	15.8	25.2	27.4	12.9	12.4

[a] Corn lines: Whorl juice (S) SC229, SC229 × Tx601, Ab24E × Va35; (R) Mp706, Mp706 × Mp707, Mp704 × Mp78.518. Lyophilized whorl (S) Ab24E × Mp305, SC229 × Tx601; (R) Mp703 × Mp704, Mp704 × MP707. Callus (S) Ab24E × Va35, Tx601 × SC229, Ab24E × Mp305; (R) Mp703 × Mp704, Mp703 × Mp707, Mp704 × Mp706. Kernels (S) Va35, Tx601, Ab24E; (R) Mp701, Mp704, Mp706.

[b] Analysis of tryptophan was obtained only from casein. Inclusion here is to indicate it was included in diets.

[c] Asparagine was determined as aspartic acid in lyophilized whorl and callus samples. For dietary purposes, it was included at a ratio to aspartic acid based on the free whorl juice amino acids.

TABLE 2. WHORL JUICE CHOICE BIOASSAYS: RESPONSES OF FAW LARVAE TO SUGARS AND AMINO ACIDS

Sample	Insects responding to test and check[a]
S × S sugars vs. check	1.8 ± 0.3
S × S amino acids vs. check	10.5 ± 1.3
R × R sugars vs. check	0.8 ± 0.2
R × R amino acids vs. check	6.3 ± 0.4
S × S sugars vs. R × R sugars	(−)0.4 ± 0.2
S × S amino acids vs. R × R amino acids	2.6 ± 0.3
S × S sugars vs. S × S amino acids	(−)4.1 ± 0.3
R × R sugars vs. R × R amino acids	(−)7.8 ± 0.4

[a] Averages for three S × S and three R × R crosses, all tests at 7 mg per water check; number responding to test sample minus check, or test sample minus other designated test sample.

amino acid fractions from both S and R whorls were much more stimulatory than the sugars, both in tests against water checks and in confrontational tests where choices were forced between sugars and amino acids. The S amino acid fraction was 42% more stimulatory than the R amino acid fraction in the confrontational tests.

Because the sugars had been found to be present in much higher concentrations than amino acids in juice, another test was conducted in which sugars were tested at 48 mg/disk while the amino acids remained at 7 mg/disk. Again, sugars were found to be only slightly stimulatory, while the amino acids were much more stimulatory. The larvae preferred amino acids to sugars in confrontational tests, and the S amino acid fraction was preferred over the R amino acid fraction.

Choice Bioassays with Commercially Procured Amino Acids and Carbohydrates. Next, 21 amino acids, three sugars, and phytic acid were evaluated as feeding stimulants in the disk bioassay to determine whether authentic amino acids could account for the stimulation of the whorl amino acid fractions. The results (summarized in Table 3) indicate that all the essential amino acids (generally the same 10 are essential for rats and insects) and all the nonessential amino acids except aspartic acid were about as stimulatory as the corn whorl amino acid fraction. On the other hand, glucose and fructose (the two free sugars found present in whorl juice) were only minimally stimulatory, and sucrose and phytic acid were mildly stimulatory.

Preference Tests with Amino Acid Mixtures. Because the corn whorl amino acid fraction and individual amino acids were stimulatory, it was of interest to test amino acid mixtures based on HPLC amino acid analyses of the S and R

TABLE 3. CHOICE BIOASSAYS WITH FAW LARVAE RESPONDING TO AUTHENTIC AMINO ACIDS AND SUGARS[a]

Compound	Response rank (test minus check)
Essential amino acids	
arg, his, i-leu, lys, met, phe, val,	10–15
thr, try, leu	7–9
Nonessential amino acids	
ala, gly, ser	10–15
asp-NH_2, cys, glu, pro, tyr	5–9
asp	<1
Sugars	
Glucose	1.0
Fructose	1.2
Sucrose	6.0
Phytic acid	5.2

[a] All tests employed 7 mg/disk.

juices (Table 1). The average number of larvae responding at the 7 mg/disk level (test minus check) were S = 13.7 $\pm$ 2.0 and R = 7.7 $\pm$ 0.9. In confrontational tests, the average response was: S − R (responding to S minus responding to R) = 5.3 $\pm$ 0.7. These results establish that the amino acids in the whorl amino acid fractions exert a major influence in initiating larval feeding, and that the S amino acids are more stimulatory.

Next, amino acid mixtures were formulated that were based on the content of only the essential amino acids in whorl juice (S and R). Similarly, other amino acid mixtures were formulated that were based on the nonessential amino acids in whorl juice (S and R). A similar set of formulations was prepared that was based on the amino acid analyses for S and R freeze dried whorls.

Mixtures based on the analysis of the total amino acids of S tissues were significantly more stimulatory than those based on R tissues. Mixtures based on the analysis of the nonessential amino acids of S tissues were also significantly more stimulatory than those based on R tissues, but mixtures based on the analysis of the essential amino acids of S tissues were not significantly more stimulatory than those based on R tissues (Table 4).

The amino acid analysis data of Table 1 show that the percent contents for the essential amino acids in S and R whorl juice, whorls, and callus tissues are not significantly different. Data in Table 3 show that three of the essential amino acids (threonine, tryptophan, and leucine) are not quite as stimulatory as the other seven, but their relative distributions in S and R tissues are not significantly different. Therefore, the finding that mixtures based on S and R essential amino acids are equally stimulatory (Table 4) is predictable.

TABLE 4. CHOICE BIOASSAYS EMPLOYING WHORL JUICE- AND FREEZE-DRIED WHORL-BASED AMINO ACIDS

Formulation	Response, test minus check[a]	
	Susceptible source	Resistant source
Whorl juice amino acids	9.5 ± 2.1	4.3 ± 0.9
Whorl juice amino acids, nonessential amino acids only	3.0 ± 0.6	1.7 ± 0.6
Whorl juice amino acids, essential amino acids only	4.6 ± 1.2	3.8 ± 1.0
Freeze-dried whorl amino acids	14.5 ± 1.3	11.0 ± 0.6
Freeze-dried whorl amino acids, nonessential amino acids only	6.1 ± 0.8	3.9 + 0.4
Freeze-dried whorl amino acids, essential amino acids only	7.3 ± 1.1	6.2 ± 0.8

[a] Average of three S and three R lines (see Table 3), all tests at 7 mg/disk.

However, there are some differences in the distribution of some of the nonessential amino acids in S and R tissues (Table 1). While the contents of the most stimulatory nonessential amino acids are similar in S and R tissues, asparagine and proline are higher in S tissues while aspartic acid and tyrosine are higher in R tissues. Because aspartic acid is the only amino acid that does not stimulate feeding, and the ratio of the more preferred asparagine to aspartic acid is higher in S lines, it is speculated that an asparagine synthetase could control that equilibrium.

One additional preference bioassay was carried out to determine whether known compounds were as stimulatory as whorl juice in confrontational tests using equal concentrations (Table 5). Formulated whorl juice amino acid

TABLE 5. CHOICE BIOASSAYS COMPARING S AND R WHORL JUICES WITH S AND R WHORL JUICE-BASED AMINO ACID (WJAA) MIXTURES AND SUGARS

Formulations	Bioassay; insects responding to tests
WJAA (S × S)[a] vs. juice (S × S)[b]	11.4/15.4
WJAA (R × R) vs. juice (S × S)	11.7/15.5
WJAA (S × S) + FRU + GLU[3] vs. juice (S × S)	12.6/13.5
WJAA (R × R)[b] + FRU + GLU vs. juice (S × S)	9.3/12.8
WJAA, essential amino acids only (R × R) vs. WJAA (R × R)	13.7/ 5.8
WJAA (S × S) vs. WJAA (R × R)	8.6/ 5.2

[a] Mixtures based on average of three S and three R lines (see Tables 3 and 6), all tests at 7 mg/disk.
[b] S × S juice (all tests) = TX 601 × T-232, R × R juice (all tests) = MP 704 × Mp 707.
[c] 7 mg fructose + 14 mg glucose/disk.

(WJAA) mixtures (S and R) were 74% and 75% as stimulatory as the comparable juices. When fructose and glucose were added to the amino acids, the mixtures were 93% (S) and 73% (R) as stimulatory as the comparable juices. A further demonstration of the decreased preference of the R nonessential amino acids is shown by the much decreased responses by FAW larvae to amino acid mixtures based on the total R WJAA as compared with that of the R essential WJAA, 5.8/13.7; 42% (Table 5). In confirmation of previous tests (Table 1), S whorl juice amino acids were 65% more stimulatory than R whorl juice amino acids. We, therefore, conclude that the stimulatory capability of the juice can primarily be accounted for by the amino acids, but the sugars complement them.

Search for Peptides in Juice and Evaluation by Choice Bioassays. Peptides are known to display various biological activities; therefore, a chromatographic analysis of the juices was performed using Sephadex G-10-120 and G-50-150 columns, both 5 × 77 cm, and employing glutamic acid and glutathione as chromatographic markers. The previously isolated corn whorl amino acids were rechromatographed on the Sephadex G-50-150 column to determine whether peptides were present. However, the elution pattern was consistent with that expected for amino acids only.

Larval Growth Studies

Dietary Tests with Freeze-Dried Whorls. Larvae fed on three susceptible hybrid lines weighed more than those on three resistant lines. The weights in milligrams with mean separation letter designations were: Ab24E × Mp305 (S × S) 389.8 a, Ab24E × Tx601 (S × S) 309.5 b, SC 229 × Tx601 (S × S) 201.4 c, Mp702 × Mp705 (R × R) 144.2 d, Mp701 × Mp 707 (R × R) 112.4 d (means followed by the same letter are not significantly different at the $P = 0.05$ level.) With this test design, it is not possible to decide whether the poorer growth on the R lines is due to the presence of toxins or antifeedants, or, alternatively, the absence of stimulants or nutritional factors. However, the bioassay does show that differences between freeze-dried tissue of resistant and susceptible hybrids are manifested in the differences in larval growth.

A second test was conducted to determine whether corn plant tissue contains a toxin in which freeze-dried whorls were added to the standard laboratory diet. Using two S inbred lines, SC213 and GT106, and two R inbred lines, Mp702 and Mp706, 10, 20, and 30 g of the powders were added to 300 ml of the normal diet. Alphacel, 0, 10, and 20 g, and 150 ml of H_2O were added to maintain suitable diet consistency.

While the addition of whorl powder to the standard laboratory diet caused decreases in larval growth, presumably because whorl powder is a poorer nutritional source, R genotypes did not depress growth below that of S genotypes at any level. At seven days, larval weights were 79.1%, 64.6%, and 61.5% of

that of insects fed the standard diet (115.8 g) when 10, 20, and 30 g of S powder (average of 2) was added, while larval weights were 81.4%, 50.0%, and 53.1% of that of insects fed the standard diet in similar tests with R powder. Relationships were unchanged at 10 days.

In this second test, the inclusion of the standard casein–wheat germ diet ensured that adequate resources for growth were available. Thus, while decreased growth on both diets might be attributed to a dilution of the nutritional resources, decreased growth on the R powder relative to the S powder presumably could be attributed to toxins in the R powder or some favorable factor in the S powder. Failure of the R powder to decrease the growth to a level lower than that of insects fed the S powder is presumptive evidence that no toxin (or growth inhibitor) was acting.

Evaluations of Nutritional Adequacy of Synthetic Amino Acid Diets. Because amino acids appeared to be the most important factor involved with preference, an investigation of the effects of the amino acid content (free and bound) in dietary sources on FAW larval growth seemed appropriate. No report could be found of previous attempts to rear FAW larvae on amino acid diets, although numerous other insects have been successfully reared on such diets.

Initially, amino acid diets were formulated by substituting amino acids for the protein (2.59% of wet weight) contributed by the casein in the standard laboratory diet. When efficacy was established, other diets based on the amino acid contents (Table 1) in whorl juice, freeze-dried whorls, and corn callus were also formulated and evaluated.

In the first dietary test, larval growth was determined for insects that were fed the standard casein–wheat germ laboratory diet, and for insects fed amino acid diets based on the casein amino acid analysis. At the same 2.59% of diet, those insects on the amino acid diet grew more slowly (18.8 ± 7.5 vs. 82.5 ± 30.2 mg at seven days), but they reached the pupal stage just four days later than those on the standard diet, at which time the weights of larvae fed the amino acid diet were comparable to those fed the standard diet, and they appeared normal. The four-day growth lag can perhaps be explained by the need for insects to develop mechanisms to transport and metabolize the free amino acids. Insects fed either 50% less or 50% more amino acids did not grow as rapidly (7.4 ± 5.5 and 10.5 ± 6.8 mg at seven days).

The somewhat poorer growth observed on the diet with 50% more amino acids probably can be explained by the high amino acid–carbohydrate ratio. Phytophagous insects have been reported to grow best on diets in which the protein and carbohydrates each contribute about one third of the nutrients (Dadd, 1985), and this ratio is approximated in the standard laboratory diet. Insects developed to normal pupae on a diet from which proline was excluded, evidence that proline, although essential to some insects (Dadd, 1985), is not essential for growth of the FAW.

The amino acids in wheat germ (analysis in Table 1) did not support growth. Larvae fed the wheat germ-based amino acid diet lived for several days, but did not grow (0.6 mg at 10 days). An amino acid diet based on the amino acid contributions of the standard casein and wheat germ (81 : 19) diet supported larval growth and development, but the larvae did not grow as rapidly (11.0 ± 6.3 g) as larvae fed the casein-based amino acid diet (18.8 ± 7.5 g) at seven days. An inspection of the amino acid distribution in wheat germ (Table 1) suggests that arginine is too high, and methionine too low, relative to the other essential amino acids.

To determine whether the reported tryptophan level (1.5% of total amino acids) was adequate for larval growth, a test was added in which the tryptophan was increased threefold. There was no increase in the growth rate (13.3 ± 5.4 mg at seven days), but evidently the higher level was not toxic, either.

To further evaluate the efficacy of synthetic amino acid diets, both the standard laboratory diet and the casein-based amino acid diet (each at 2.59% amino acids) were fed to FAW larvae and weighed initially, and at 4, 7, 10, 12, and 15 days or until they pupated.

The results (Table 6) show that larval growth was slower on the amino acid diet, but that these insects attained weights nearly as great as those fed the standard diet. They required 4.2 additional days to reach pupation. Slightly greater numbers (but only 8%) of the adults possessed "slight abnormalities" and pupal weights were about 14% lower. Nevertheless, the performance of larvae on the amino acid diets can be considered near normal and, presumably, could be further optimized if desired.

TABLE 6. GROWTH AND DEVELOPMENT OF FAW LARVAE ON STANDARD LABORATORY AND SYNTHETIC AMINO ACID DIETS; Mg, X ± Sd.[a]

Age (days)	Standard diet (mg)	Amino acid diet (mg)
0	0.06[b]	0.06
4	7.6 ± 2.6	2.0 ± 0.5
7	103.2 ± 34.5	18.1 ± 7.1
10	553.0 ± 93.1	106.6 ± 31.8
12		224.6 ± 67.4
15		537.9 ± 65.6
Pupae	297.2 ± 37.9	255.2 ± 30.4
Days to pupation	13.6 ± 0.8	17.8 ± 1.0
Pupae to adults	9.1 ± 0.9	9.8 ± 0.8
Slightly abnormal adults (%)	3	8
Severely abnormal adults (%)	0	0

[a] Amino acids or protein amino acids constitute 2.59% of the diet on a wet weight basis.
[b] Ng (1988).

Dietary Tests with Whorl Juice-Based Amino Acids Mixtures and Whorl Juice. Larvae fed amino acid diets at the 2.59% level based on the S and R whorl juice amino acids did not grow and died after a few days. Failure to grow is attributed to the low content of essential amino acids (11.2% and 13.0% of the total amino acids). Next, the freeze-dried brown-band fractions from S and R whorl juices were incorporated in diets as the only source of amino acids at 2.59% so that the essential amino acids were present at S = 0.29% and R = 0.34% of the total diet, wet weight basis. The FAW larvae did not grow, so that it can be concluded that while the whorl juice amino acids are adequate to stimulate feeding, they lack sufficient quantities of the essential amino acids to support growth.

Dietary Tests with Freeze-Dried Whorl-Based Amino Acid Mixtures. Larvae fed amino acid diets at the 2.59% level based on the S and R freeze-dried whorl amino acids grew as rapidly (16.5 $\pm$ 10.6 and 17.8 $\pm$ 11.2 mg at seven days, respectively) as those on the casein-based amino acid diet (17.3 $\pm$ 8.9 mg at seven days), and they later achieved normal pupal weights and characteristics. Evidently, even though the R amino acids include greater amounts of less-preferred nonessential amino acids, they do not cause decreased larval growth. This may be so because the levels and ratios of essential amino acids in S and R whorls are similar (Table 1).

In a demonstration test, two amino acid mixtures based on S freeze-dried whorls, but from which lysine and methionine in turn were omitted and the total concentration readjusted to 2.59%, were evaluated. As expected, the larvae failed to grow, and subsequently died in a few days, showing that these two essential amino acids cannot be withheld if larvae are to grow. It is assumed that withholding any of the other eight essential amino acids would also prevent larval growth (Dadd, 1985).

In another test, seven freeze-dried whorl-based amino acid diets of increasing content (0.57–3.97%, wet weight basis) were fed to FAW larvae, and weights were recorded to determine the optimum dietary amino acid level and, by inference, to determine whether the standard laboratory level (2.59%) was optimal. Growth at the 2.27% amino acids level was optimal, and growth decreased appreciably above 3.4% amino acids. Growth at the 1.7% amino acid level was nearly optimal (93%). The growth rate varied with $Y = -8.81 + 3.67\ X - 0.13\ X^2$ where Y is larval weight and X is the percent total amino acids. Thus, the 2.59% amino acid content of the standard diet can be considered nearly optimal.

Dietary Tests with Corn Callus-Based Amino Acid Mixtures. Techniques for growing corn callus tissue have been developed at this location (Williams et al., 1983). With callus tissues it has been shown that FAW larvae are stimulated more strongly to feed on S lines than on R lines (Williams et al., 1985). Also, FAW larvae fed for seven days on S corn callus were larger (S average, three lines: 26.5 mg) than those fed on R lines (R average, four lines: 16.9

mg). Amino acid analyses (Table 1) and larval growth tests at 2.59% amino acids were conducted to evaluate the nutritional resources of callus tissue. Compared to larvae fed on the casein-based amino acid diet (13.5 ± 4.3 mg at seven days), growth was somewhat slower, but successful, on S and R callus-based diets at the same total amino acid level (S = 8.6 ± 4.2 and R = 8.9 ± 4.2 mg). This can be attributed to the lower callus essential amino acid content (S = 35.2%, R = 33.5%) than that of the casein amino acid diet (46.8%).

Because no differences could be demonstrated using S and R callus-based amino acid diets in the larval growth tests, the greater growth on S callus may be attributed presumptively to differences in preference, perhaps differences in the nonessential amino acids (Table 1).

Analyses of Corn Tissues

Analysis of Protein and Fiber in Freeze-Dried Whorls. Repeated analyses for percent whorl protein in this and our previous study (Hedin et al., 1984) have shown 15–30% higher levels in S tissues than in R tissues. Table 7 gives the results of an analysis in which the whorl was divided into four sections, B and C being the sites of greatest larval feeding. The average protein content of the S varieties was about 17% higher than the R varieties in this test.

However, this difference is evidently not large enough to be manifested in larval growth tests. In our test with seven freeze-dried whorl-based amino acid diets, larval growth was found to be at a maximum over a fairly wide range (1.7–3.4% dry weight). Percent protein values for both S and R tissues are within, or close to, this range. If there is an advantage to R plants to biosynthesize less protein, larval growth tests do not provide an explanation.

In our previous study (Hedin et al., 1984), we also found that S varieties were 12–30% lower in crude fiber and in 70% aqueous methanol-extracted fiber. Table 8 gives the results of an analysis of the residue contents of a number of S and R genotypes. Again, the residue content of the R genotypes was higher than that of the S genotypes. Unfortunately, incorporation of fiber, including alphacel and corn cob grits, at levels reflecting the differences in plant tissue has not given measurable differences in larval growth tests (Hedin et al., 1984). Of course, this test design cannot reflect the histological environment encountered by the insect where tissue toughness may discourage or slow feeding.

Using AOAC procedures 7.055, 7.056, and 7.057 on two S and two R varieties, analyses were made for soluble and bound protein, cell contents, and cell wall contents (Horwitz, 1975). For the S varieties, protein (all soluble) was 20.6%, total cell contents 45.7%, and cell wall contents 54.3%. For the R varieties, protein (all soluble) was 16.1%, total cell contents 40.4%, and cell wall contents 59.6%. S varieties contained more protein, as reflected by lower cell wall protein, and less fiber, as reflected by lower cell wall contents. Hemi-

TABLE 7. PERCENT PROTEIN OF FREEZE-DRIED WHORL TISSUE FROM S × S AND R × R CORN GENOTYPES[a]

Sample	Tissues from S × S (%)				Sample	Tissues from R × R (%)			
	A	B	C	D		A	B	C	D
Ab24E × Mp305	19.4	19.1	19.8	21.3	Mp704 × Mp707	15.8	15.2	15.6	16.4
SC229 × Tx601	17.9	18.1	18.1	17.9	Mp496 × Mp701	15.1	15.4	14.6	14.9
B86 × Va35	18.8	17.9	17.7	19.1	Mp703 × Mp707	16.4	16.4		
GA172 × Mp305	19.4	17.9	18.3	18.6	Mp701 × Mp707	16.6	16.6	15.8	16.8
C21 × Ga203	19.7	18.9	19.4	18.8	Mp702 × Mp705	15.9	15.8	16.4	17.6
Ab24E × Va35	17.8	18.3	18.8	18.4	Mp703 × Mp704	15.2	15.6	17.2	18.9
Mean	18.8	18.4	18.7	19.0	Mean	15.8	15.8	15.9	16.9
Var	0.6	0.2	0.5	1.2	Var	0.3	0.3	0.8	1.8
Std Err	0.3	0.2	0.3	0.5	Std Err	0.3	0.2	0.4	0.7
S × S (% of R × R)	119.0	116.5	117.6	112.4					

[a]Whorls from plants at V_8–V_{10} stages of growth were sectioned into A, yellow tissue; B, yellow-green; C, green-yellow; and D, green tissue.

TABLE 8. PERCENT RESIDUE REMAINING FROM 70% AQUEOUS METHANOL EXTRACTION OF FREEZE-DRIED WHORL TISSUE FROM S × S AND R × R CORN GENOTYPES[a]

	Tissue from S × S (%)					Tissue from R × R (%)			
Sample	A	B	C	D	Sample	A	B	C	D
C21 × Ga203	60.2	65.8	71.6	74.3	Mp704 × Mp707	72.4	70.7	76.0	75.8
B86 × Va35	60.5	68.4	73.6	74.7	Mp702 × Mp705	68.2	74.8	80.0	74.0
Ab24E × Mp305	64.3	64.7	68.0	65.5	Mp496 × Mp701	70.6	73.0	71.2	75.4
SC 229 × TX 601	64.5	68.0	69.4	70.6	Mp701 × Mp707	74.5	77.0	79.2	76.2
Ga172 × Mp305	60.3	59.9	64.0	67.2	Mp703 × Mp706	71.5	74.5	79.2	76.9
GT106 × Mp462	64.1	71.4	68.7	71.8	Mp703 × Mp707	74.0	76.4	75.2	76.9
Ab24E × Mp462	59.8	62.8	68.2	69.7	Mp703 × Mp704	77.9	78.5	78.3	78.2
Ab24E × Tx601	68.7	73.6	69.9	68.0					
Ab24E × Va35	59.1	67.2	74.3	74.6					
Mean	62.4	66.9	69.7	70.7	Mean	72.7	75.0	77.0	76.2
Var	10.1	17.6	9.8	11.6	Var	9.4	6.6	9.4	1.7
Std Err	1.1	1.4	1.1	1.1	Std Err	1.2	1.0	1.2	0.5
R × R residue (% of S × S)	116.6	112.2	110.4	108.0					

[a]Whorls from plants at V_8–V_{10} stages of growth were sectioned into A, yellow, B, yellow-green; C, green-yellow; and D, green tissues.

cellulose and lignin were lower in S lines, while cellulose was unchanged confirming results obtained in the earlier study (Hedin et al., 1984).

Analysis of Maysin in Whorls and Silks. Maysin has been reported as a factor in resistance of the corn genotype Zapalote Chico to the corn earworm (Waiss et al., 1979). Therefore, silks from this genotype, from Stowell's Evergreen (known to be low in maysin), and from silks and whorls of two of our genotypes, one susceptible to the FAW, and another resistant, were analyzed for maysin. Percent content of maysin on a wet weight basis for the corn tissues were as follows: silks—Zapalote Chico = 0.909, Stowell's evergreen = 0.005, SC229 × Tx601 = 0.105, and Mp496 × Mp701 = 0.145; whorls: SC229 × Tx601 (S × S) = 0.0008, and Mp496 × Mp701 (R × R) = 0.0022.

These analyses confirm the reported presence of maysin in Zapalote Chico, but show that it is present in a very low concentration in silks and whorls of our experimental lines. It appears unlikely, therefore, that maysin is a significant factor of resistance in our resistant lines to the FAW.

SUMMARY

Evidence has been developed that feeding in the whorl by young FAW larvae is stimulated by whorl juice amino acids to a much greater extent than by sugars, and stimulatory effects by other whorl juice constituents appears to be limited. The percent content of essential amino acids in the whorl juice, S or R, however, is too low to support larval growth. Aspartic acid is the only amino acid that does not stimulate feeding, and the ratio of more preferred asparagine to aspartic acid is higher in S lines (Table 1). It is speculated that an asparagine synthetase could control that equilibrium.

Larval growth studies showed that amino acid diets based on the amino acid analysis of protein sources such as casein, freeze-dried whorl tissue, and callus tissue support growth effectively. After a four-day lag period, during which the young larva evidently adapts metabolically to the free amino acid source, growth proceeds to that of full-sized pupae of normal characteristics. There were no differences in growth when diets based on S and R sources were employed at the same levels, the essential amino acids of both evidently being adequate. Proline, asparagine, and aspartic acid all have been identified as essential amino acids for some insects (Dadd, 1985), but larval growth tests do not establish their essentiality for the FAW.

The achievement of successful growth of FAW larvae fed free amino acid diets to normal adulthood is noteworthy, as it has not been reported previously with this insect. The withholding of even one essential amino acid from these diets prevented growth, as expected, and the larvae died within a few days.

When freeze-dried whorl tissue was provided to larvae as agar–water plugs, those fed the S tissue grew larger but no mechanism could be inferred because

the differences could be attributed either to a stimulant in the S tissue or the presence of a toxin in the R tissue. In a second test where the whorl tissue was added to the standard diet to achieve nutritional adequacy, there was no difference in growth on S and R tissues, making the presence of a toxin unlikely. Neither 6-MBOA nor maysin appeared to be factors of resistance in our corn whorls because they are present only in very small quantities in whorls, and 6-MBOA is not very toxic to the FAW.

Thus, the mechanisms of resistance evidently are those related to nonpreference and difficulty encountered in feeding because of higher fiber. The significance of these findings is that analysis of individual amino acids, protein, and fiber may provide a basis for the selection or genetic production of corn lines resistant to the FAW. Another favorable finding is that the differences in protein content and individual amino acids are not carried over to S and R kernels (S = 12.9%, R = 12.4% protein) (Table 1). Thus selection for whorl resistance to insects does not unfavorably affect the nutritional value of the grain to consumers.

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A NEW COMPONENT OF THE FEMALE SEX PHEROMONE OF *Blattella germanica* (L.) (DICTYOPTERA: BLATTELLIDAE) AND INTERACTION WITH OTHER PHEROMONE COMPONENTS

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Abstract—A fourth component, 3,11-dimethyl-2-heptacosanone, was identified as a cuticular contact sex pheromone of the female German cockroach, *Blattella germanica*. In behavioral assays, higher dosages of 3,11-dimethyl-2-heptacosanone were needed to elicit similar sexual responses in males to those elicited by the major pheromone component, 3,11-dimethyl-2-nonacosanone. A 15 : 85 blend of the C_{27} and C_{29} methyl ketone homologs resulted in a dose–response curve intermediate between that of each of the components alone, indicating independence of activity of each component and lack of synergism. Moreover, the activity of 3,11-dimethyl-2-nonacosanone was not enhanced by female cuticular hydrocarbons. The relationship between sexual responses of males to females and to isolated female antennae, and the amount of cuticular pheromone on whole females was investigated. Cuticular sex pheromone found on females increased with the age of the female, as did the male response to whole females. However, a bimodal male response was elicited by isolated female antennae. Differences between behavioral and analytical assays of pheromone are discussed.

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Key Words—German cockroach, *Blattella germanica*, Dictyoptera, Blattellidae, sex pheromone, behavioral response, contact pheromone, 3,11-dimethyl-2-heptacosanone, 3,11-dimethyl-2-nonacosanone.

INTRODUCTION

The female German cockroach, *Blattella germanica*, produces a nonvolatile three-component cuticular sex pheromone. The most abundant component is 3,11-dimethyl-2-nonacosanone, with lower amounts of 29-hydroxy-3,11-dimethyl-2-nonacosanone and 29-oxo-3,11-dimethyl-2-nonacosanone (review: Nishida and Fukami, 1983). Whole females and isolated body parts of sexually mature females (e.g., antennae, legs, wings) elicit the complete courtship wing-raising response in males, as does each of the three pheromone components alone (Roth and Willis, 1952; Bell and Schal, 1980; Nishida and Fukami, 1983). Grouped females become receptive to courting males four to five days after adult emergence (Gadot et al., 1989).

Nishida and Fukami (1983) noted that excised antennae of individually isolated teneral females and of sexually mature females elicit courtship wing-raising responses in the majority of tested males, whereas antennae from young, sexually immature adult females elicit responses in only a fraction of the males tested. However, gas–liquid chromatography (GLC) of female extracts at five-day intervals suggests that cuticular pheromone in isolated females increases gradually between days 0 and 15 (Schal et al., 1990). This apparent discrepancy between analytical and behavioral observations has not been resolved because the relationship between the amount of pheromone on females at different ages and male response has not been studied. To address this, we examined the amount of cuticular pheromone found on individual females at different ages.

The major hydrocarbon component, from GC-MS analysis of sexually mature *B. germanica* females, is an isomeric mixture of 3,7-, 3,9-, and 3,11-dimethylnonacosane (Augustynowicz et al., 1987; Carlson and Brenner, 1988; Jurenka et al., 1989). Jurenka et al. (1989) and Chase et al. (1990) suggested that the production of the methyl ketone pheromone component results from the sex-specific oxidation of its hydrocarbon analog. Since a mixture of isomers of 3,9- and 3,11-dimethylheptacosane also is found in cuticular washes of females (ca. 2% of the total hydrocarbons) but only the 3,11-dimethyl ketone isomer (3,11-dimethyl-2-heptacosanone) was found (Jurenka et al., 1989), and since neither methyl ketone homolog was found in adult males, we hypothesized that 3,11-dimethyl-2-heptacosanone might act as a fourth pheromone component. We report here on the verification of this hypothesis using behavioral assays and on the responses of males to combinations of the C_{27} and C_{29} methyl ketone components.

METHODS AND MATERIALS

Insects. Cockroach nymphs that hatched within four days of each other were reared in 2-liter glass jars and fed dry Purina dog chow and water. Newly emerged (day 0) adult males and females were separated daily and kept in groups until use. Both nymphs and adults were kept at 27°C under a 12 : 12 light–dark photoperiod.

Extraction, Separation, and Quantification of Pheromone. Cuticular lipids of females (10 per group) were extracted with two 3-ml hexane washes, each for 5 min. Two internal standards, 14-heptacosanone (100 ng) and 1-hexacosanol (45 ng), were included during extraction to allow quantification by GLC. The hexane washes were combined and most of the solvent removed under N_2. The extracts were separated on TLC plates (silica gel 60F-254; EM Science) developed twice in hexane–diethyl ether (93 : 7 v/v). Fractions were scraped into test tubes and extracted with diethyl ether.

The fraction containing 29-hydroxy-3,11-dimethyl-2-nonacosanone was reduced to dryness under N_2, and 100 μl redistilled acetyl chloride was added for 30 min to acetylate the pheromone component. Samples were analyzed on a HP 5890A GLC equipped with a splitless injector and a flame-ionization detector, and interfaced with a HP 3390A integrator. Injection in isooctane or methylene chloride (methyl ketone and hydroxy methyl ketone, respectively) was made onto a 15 m × 0.53 mm ID SPB-5 column programmed from 60°C to 250°C at 20°C/min and then to 320°C at 3°C/min. The injector and detector were maintained at 330°C.

Test Compounds. Synthetic racemic 3,11-dimethyl-2-nonacosanone (80% pure, 1.9% 3,11-dimethyl-2-heptacosanone) and 29-hydroxy-3,11-dimethyl-2-nonacosanone were obtained from Drs. A.W. Burgstahler and R. Nishida. The natural pheromone components have the (3*S*,11*S*) configuration, but all the synthetic stereoisomers are active at the same concentration in behavioral assays (Nishida and Fukami, 1983). Other compounds were obtained from extracts of sexually mature females and separated by TLC as described above. The C_{27} and C_{29} methyl ketone components (85% of the methyl ketone fraction; 15% unknown compounds) were separated by GLC and cold-trapped in 30-cm capillary glass tubes using the GLC procedure described above. Each was more than 93% pure with less than 0.5% of the other homolog.

The highest concentration of each test compound or blend for behavioral assays was calibrated by GLC against an external standard and then serially diluted in CCl_4.

Bioassay. Male response was tested using a modification of the assay developed by Roth and Willis (1952) and adopted by Nishida and Fukami (1983). An antenna of a male *Supella longipalpa* cockroach was excised and

mounted on a glass Pasteur pipet and a 5-μl solution of test compound in CCl_4 was applied to its distal 1 cm. The antenna was used immediately to test the sexual responses of 30 males 14–30 days old that were housed individually in 9-cm-ID × 5-cm-deep glass beakers and supplied with dog food and water. A positive response consisted of turning away from the stimulus and raising the tegmina and wings to a 90° angle within 30 sec. Any male that failed to respond to both a test compound and later to the antenna of a six-day-old female was discarded. Since isolated males are most responsive in the scotophase (Bell et al., 1978), bioassays were conducted hourly between hours 2 and 10 of a 12-h scotophase. The results were subjected to a maximum likelihood probit-log dose analysis (SAS, 1985).

All treatments were systematically arranged so that low, medium, and high dosages were equally represented in the early, middle, and late portions of the scotophase.

To compare male responses elicited by isolated female antennae (above) to the responses elicited by whole females, single live females were introduced successively to two individually isolated males. The latency between contact with the male and male wing-raising was recorded. Males were allowed 30 sec to respond. A total of 54 males were tested using 27 females. The same cohort of females was used daily for seven days between the ages of 0 and 6 days.

RESULTS

3,11-Dimethyl-2-heptacosanone: A New Pheromone Component. We used *Supella longipalpa* antennae to deliver test compounds to males in order to avoid any confounding effects of conspecific odors. Isolated *B. germanica* males did not respond to excised antennae of male *S. longipalpa* mounted on Pasteur pipets. However, when loaded with synthetic 3,11-dimethyl-2-nonacosanone, or when rubbed against the cuticle of a sexually receptive *B. germanica* female, such antennae elicited in the male a characteristic stilt posture, antennal waving, an abdominal twitch, and a rotational turn accompanied by wing-raising (see Roth and Willis, 1952; Bell and Schal, 1980). Courtship wing-raising exposes a tergal gland on the male, which the female palpates, placing her in position for copulation. Wing-raising is a terminal act of the male's sexual response requiring chemosensory stimuli, and subsequent acts such as copulatory thrusts can be elicited in males by physical stimuli alone applied to its tergum (mimicking female mounting).

Both synthetic and female-extracted, GLC-separated 3,11-dimethyl-2-nonacosanone elicited similar responses in *B. germanica* males (Figure 1A). Overlapping fiducial limits at the RD_{50} and RD_{75} and similar slopes indicated identical dose–response curves for both materials (Table 1). Female-extracted and GLC-separated 3,11-dimethyl-2-heptacosanone also elicited strong sexual responses

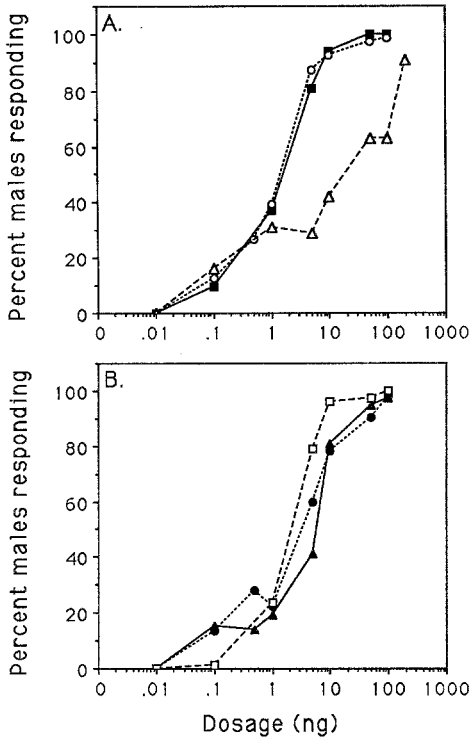

FIG. 1. (A) Responses of *B. germanica* males to various dosages of natural (circles) and synthetic (squares) 3,11-dimethyl-2-nonacosanone and natural 3,11-dimethyl-2-heptacosanone (triangles). At least 75 males were tested with each compound at each dosage, except at 200 ng at which $N = 30$ males. (B) Responses of *B. germanica* males to various dosages of the TLC-purified methyl-ketone fraction (circles), a 15:85 blend of natural GLC-separated 3,11-dimethyl-2-heptacosanone and 3,11-dimethyl-2-nonacosanone (triangles), and 2.5 μg TLC-purified cuticular hydrocarbons combined with synthetic 3,11-dimethyl-2-nonacosanone (squares). At least 76 males were tested with each combination at each dosage.

in males. However, the dose–male response relationship with 3,11-dimethyl-2-heptacosanone was significantly different from both the natural and synthetic 3,11-dimethyl-2-nonacosanone, based on nonoverlapping fiducial limits following probit analysis (Figure 1A, Table 1).

Interactions among Pheromone Components. The methyl ketone TLC fraction from extracts of sexually mature females (20% 3,11-dimethyl-2-heptacosanone, 64% 3,11-dimethyl-2-nonacosanone) elicited intermediate responses between each of its two major components (Figure 1). The RD_{50} dosage was not significantly different from that of 3,11-dimethyl-2-nonacosa-

TABLE 1. MAXIMUM LIKELIHOOD PROBIT ANALYSIS OF SIX LOG-DOSAGE–MALE RESPONSE RELATIONSHIPS

Treatment	Dosage (ng)		Slope
	RD_{50}[a] (95% FL)	RD_{75} (95% FL)	
3,11-Dimethyl-2-nonacosanone			
Synthetic	1.2 (0.9–1.5)	3.4 (2.6–4.5)	1.47
Natural	1.1 (0.7–1.8)	3.7 (2.3–6.6)	1.31
3,11-Dimethyl-2-heptacosanone	17 (7.0–58)	392 (99–7409)	0.50
Methyl-ketone TLC fraction	2.2 (1.2–3.7)	10 (5.9–22)	1.00
15:85 C_{27}:C_{29} methyl ketones	3.2 (1.0–10)	13 (4.7–94)	1.11
3,11-Dimethyl-2-nonacosanone + 2.5 μg hydrocarbons	2.0 (0.9–3.5)	4.5 (2.6–10)	1.88

[a] RD_{50} and RD_{75}: dosages required to elicit wing raising responses in 50% and 75% of tested males, respectively. FL: 95% fiducial limits (in nanograms) associated with the 50% and 75% response levels.

none alone, but significantly higher dosages of the whole methyl ketone fraction were required to elicit responses in 75% or more of the cockroaches (Table 1). These data suggest that the less active 3,11-dimethyl-2-heptacosanone is responsible for the reduced activity of the total methyl ketone fraction. Combining the two major C_{27} and C_{29} methyl ketone pheromone components in a 15:85 ratio resulted in a dose–response curve identical to that obtained with the total methyl ketone fraction (Figure 1B, Table 1). Synergism was clearly not indicated, and it is unlikely that other minor components in this TLC fraction significantly enhance or suppress male responsiveness.

Various amounts of synthetic 3,11-dimethyl-2-nonacosanone and 2.5 μg cuticular hydrocarbons (TLC fraction) were loaded onto *S. longipalpa* male antennae. An antenna of a 6-day-old *B. germanica* female contains 1087 ± 32 ng (mean ± SEM, $N = 5$) of hydrocarbons. The dose–response relationship to these mixtures was not significantly different from the response to 3,11-dimethyl-2-nonacosanone alone (Figure 1B, Table 1).

Behavioral Responses of Males to Females and to Isolated Female Antennae. Our assays employed grouped females, which undergo sexual maturation faster than isolated females (Gadot et al., 1989), while Nishida and Fukami (1983) used isolated females. We therefore repeated the dose–response relationship study with antennae of different aged grouped females. Our results clearly corroborate Nishida and Fukami's (1983): Antennae from newly emerged females elicited responses in nearly 100% of tested males (ca. 85% in their assays), the response declined to 58% by day 3 (nearly 0% by day 4 in their assays) and peaked at 100% on day 6 [day 9 in Nishida and Fukami (1983)]

(Figure 2A). Male response remained at or near 100% throughout the female's adult life (data not shown).

Male responses to whole females were significantly different from responses to isolated antennae from females of the same ages (Figure 2A). Eighty percent of tested males responded within 30 sec of contacting a newly emerged female, and the response increased steadily to 100% upon contact with 5-day-old females. The latency of the male wing-raising response to whole females decreased from 8.6 sec (day 0 females) to 5.2 sec (6-day-old females) (Figure 2B). Thus, the patterns of male responses to whole females and to isolated female antennae are different.

Pheromone Accumulation over Time. Newly emerged females contained 4.4 ± 0 ng, 3,11-dimethyl-2-heptacosanone and 9.3 ± 0.8 ng 3,11-dimethyl-2-nonacosanone. In females reared in groups, the amounts of both components increased after emergence in relation to oocyte maturation (Figure 3); on average, females oviposited on day 9 and contained 187 ± 33 ng 3,11-dimethyl-2-heptacosanone and 494 ± 28 ng 3,11-dimethyl-2-nonacosanone. After day 2, the percentage composition of the two components remained the same (ca. 40:60, respectively) until ovulation. The amount of 29-hydroxy-3,11-dimethyl-

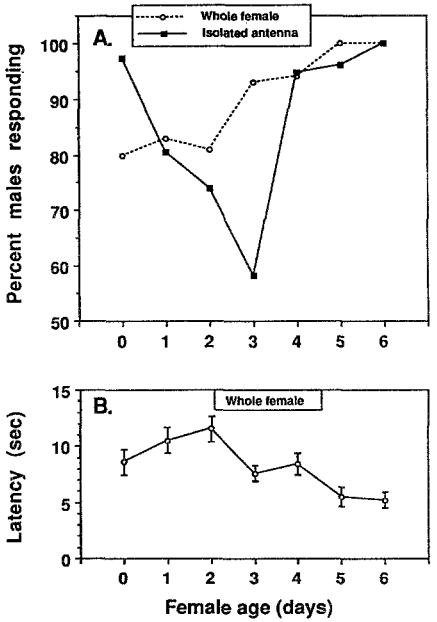

FIG. 2. (A) Percentage of *B. germanica* males (N = 54) responding within 30 sec of stimulation with whole females (N = 27) and isolated female antennae of various ages (N = 77). (B) Latency (in seconds) of male response to whole females of various ages.

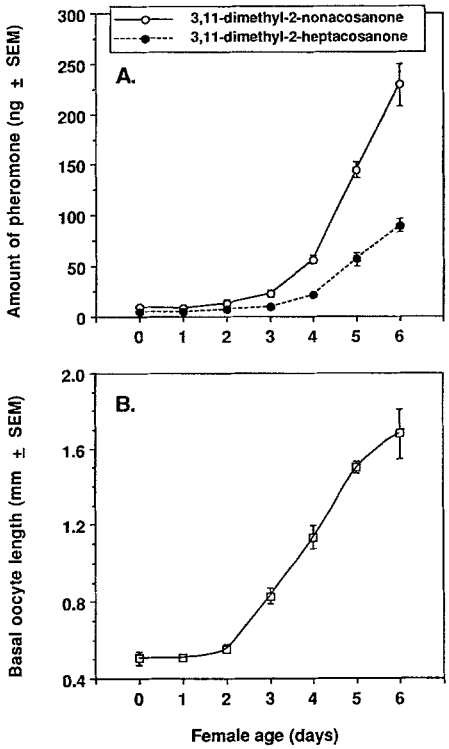

FIG. 3. (A) Amount of 3,11-dimethyl-2-heptacosanone and 3,11-dimethyl-2-nonacosanone per female of various ages. Each point represents the mean (±SEM) of three to five determinations of 10 females each. (B) Relationship between age and oocyte length in *B. germanica* ($N > 10$ for each mean).

2-nonacosanone increased from 3.2 ng per female on day 3 to 11.6 ng on day 9 (data not shown).

DISCUSSION

Sex Pheromone Blend of B. germanica. Nishida and Fukami (1983) summarized work on three components of the female sex pheromone of *B. germanica*. We now add a fourth component, 3,11-dimethyl-2-heptacosanone, to this blend. This component is second only to 3,11-dimethyl-2-nonacosanone in mass on the cuticle of sexually receptive females (Figure 3A). However, it is at least an order of magnitude less effective than 3,11-dimethyl-2-nonacosanone (Figure 1A, Table 1). The mass–activity relationship among the four components is as follows: 3,11-dimethyl-2-nonacosanone is most abundant, followed by

3,11-dimethyl-2-heptacosanone (Figure 3A), 29-hydroxy-3,11-dimethyl-2-nonacosanone and, lastly, 29-oxo-3,11-dimethyl-2-nonacosanone (data not shown). The methyl alcohol is most active, followed by the C_{29} and C_{27} methyl ketones and, lastly, 29-oxo-3,11-dimethyl-2-nonacosanone (Figure 1) (Nishida and Fukami 1983). Small amounts of unknowns in the TLC methyl ketone fraction may comprise other pheromone components. Moreover, the suggestion that the methyl ketone components are formed through oxidation of the respective branched alkanes by a 3,11-dimethyl specific enzyme (Jurenka et al., 1989; Chase et al., 1990) and presence of 3,11-dimethylhentriacontane in cuticular extracts of *B. germanica* (Carlson and Brenner, 1988), together suggest that 3,11-dimethyl-2-hentriacontanone will be another pheromone component.

The lower pheromonal activity of the C_{27}, as compared with the C_{29} methyl ketone component, is predicted from the work of Nishida and Fukami (1983): Shorter or longer carbon chains of the pheromone analog 3-methyl-2-nonacosanone had lower activity. It is expected therefore that the activity of 3,11-dimethyl-2-hentriacontanone, if found on the female's cuticle, will be lower than that of 3,11-dimethyl-2-nonacosanone.

As noted by Nishida and Fukami (1983), activity of the sex pheromone blend of *B. germanica* is strikingly different from blends in other insects (e.g., Lepidoptera). Each component alone can elicit the complete courtship wing-raising response in the male cockroach, whereas in most insects deletion of components reduces responses (review: Tamaki, 1985). In addition, combining components does not enhance or synergize their activity (Figure 1B). Indeed, the combined activity of the more active 3,11-dimethyl-2-nonacosanone and the less active 3,11-dimethyl-2-heptacosanone is intermediate between the activities of each component alone.

Blattella germanica nymphs and adult males and females have the same cuticular hydrocarbons, although their relative compositions vary (Jurenka et al., 1989). The rationale for combining 3,11-dimethyl-2-nonacosanone with female cuticular hydrocarbons on a *S. longipalpa* antenna was to verify whether a "species-specific signature" will enhance the activity of the pheromone. Clearly, this was not the case (Figure 1B, Table 1). In the housefly, *Musca domestica*, Uebel et al. (1976) reported that sex specific methyl- and dimethyl-branched C_{27} and C_{29} alkanes enhanced the mating strike activity of males when combined with (*Z*)-9-tricosene, the major sex pheromone component. Male flies possess very small amounts of methyl-branched alkanes compared with mature females (Nelson et al., 1981), and therefore the methylalkanes represent true sex pheromone components. More recent work by Adams and Holt (1987) confirmed that the C_{23} alkene is the main sex attractant, but they found that the methylalkanes promote sexual contact and mating as arrestants. Furthermore, Adams and Holt (1987) reported that a C_{23} epoxide and ketone present only on the female housefly serve as sex recognition factors. In contrast, each of the

four recognized contact sex pheromone components of the German cockroach elicits the complete courtship wing-raising response in the male cockroach.

Comparison of Analytical and Behavioral Assays of Pheromone. The pattern of pheromone production, as measured by its accumulation on the cuticle was reported previously (Schal et al., 1990). However, it was measured in isolated females at five-day intervals and was not directly related to oocyte maturation. For the present study it was important to track the daily changes in cuticular pheromone on whole females reared in groups. The data indicate that the amounts of each of the three major components gradually increase after the adult molt through at least day 6, in relation to oocyte maturation (Figure 3 and unpublished observations), and reach maximal values near or shortly after ovulation (Schal, unpublished observations). These results support our previous determinations that were conducted on isolated females every five days (Schal et al., 1990). Similar correlations of pheromone production and ovarian development were obtained with the housefly (Dillwith et al., 1983).

In contrast to these findings with whole female extracts, isolated antennae of teneral and 9-day-old sexually mature females elicited strong wing-raising responses in males, while the antennae of 4-day-old females elicited little or no response (Nishida and Fukami, 1983). In order to explain differences between behavioral and analytical results, we repeated Nishida and Fukami's (1983) behavioral assays using the same colony of cockroaches as we used for analytical determinations (Figure 2). Clearly, the pattern of behavioral responses to isolated female antennae is similar in both studies. In our work, male response declines with female age between 0 and 3 days, but subsequently increases and peaks in 6-day-old females (Figure 2A). The differences between our results and Nishida and Fukami's (1983) are likely due to different rearing and assay temperatures in the respective laboratories (27°C and 25°C). More importantly, however, our assays were conducted on grouped females whereas their work employed individually isolated females. Schal et al. (1990) showed that juvenile hormone induces production of sex pheromone in *B. germanica* females, and Gadot et al. (1989) showed that grouping accelerates the gonotrophic cycle by advancing corpora allata activity. Grouping of females would therefore advance pheromone synthesis, resulting in greater male responses to young grouped females than to isolated females of the same age.

Whole newly emerged females contain ca. eight times more 3,11-dimethyl-2-nonacosanone than is required on isolated antennae to elicit responses in 50% of tested males (Figure 1, Table 1). Although antennal fencing alone can stimulate males to perform the wing-raising response, other female body parts also are effective inducers of this response (Roth and Willis, 1952), and the male usually antennates various body parts of young females (Schal, personal observation). Therefore, the response to isolated antennae differs from that to whole

females: Responses increase from 80% to 100% using day 0 to day 5 females, respectively, with no decrease in response as seen with isolated antennae on day 3 (Figure 2A). Although the latency of male response to whole females suggests a bimodality of response, this is not a significant trend (Figure 2B).

Schal et al. (1990) suggested that the response elicited by teneral females is unrelated to the production of 3,11-dimethyl-2-nonacosanone and 29-hydroxy-3,11-dimethyl-2-nonacosanone, as this response also is elicited by teneral males and nymphs (Roth and Willis, 1952; Nishida and Fukami, 1983). The present study supports this hypothesis. Thus, the response to isolated antennae may be the net response to other stimulatory factors on teneral antennae, which dissipate over time, and to pheromone components, which increase over time (Figure 3A). The trough in the response pattern to isolated antennae disappears when whole females are tested, indicating that the amount of pheromone on the antenna of a 3-day-old female is close to the threshold of male response, but the pheromone on the rest of her body is sufficient to elicit a response. A similar situation, where males respond to teneral males and females, but where females become receptive several days later, was described in *Nauphoeta cinerea* (Schal and Bell, 1983).

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