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RELATIONSHIP OF TARBUSH LEAF SURFACE TERPENE PROFILE WITH LIVESTOCK HERBIVORY[1]

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Abstract—Tarbush (*Flourensia cernua* DC.) is a Chihuahuan Desert shrub with a resinous leaf surface containing terpenes that may affect livestock herbivory. Cattle, sheep, and goats were densely stocked in paddocks containing tarbush in two consecutive years for six to nine days and defoliation of 160 plants was recorded daily. Plants were categorized as exhibiting high or low defoliation. Leaves were collected from these plants the third year for chemical analysis. A selection procedure was used to generate two variable sets closely related to defoliation category. One set contained 14 variables (dry matter, ash, α-pinene, sabinene, 3-carene, *p*-cymene, limonene, camphor, borneol, *cis*-jasmone, β-caryophyllene, α-humulene, ledene, and flourensadiol) and the other set contained 14 unidentified compounds. When subjected to multivariate analysis, each group distinguished between the two defoliation categories ($P < 0.001$ and $P < 0.0019$ for known and unknown variable sets, respectively). These data support the hypothesis that leaf surface chemistry of individual tarbush plants is related to extent of defoliation by livestock.

Key Words—*Flourensia cernua*, leaf surface chemistry, diet selection, epicuticular wax, monoterpenes, sesquiterpenes.

INTRODUCTION

Desert grasslands are being replaced by shrubs in many arid regions of the world. This transition is often rapid and signifies serious deterioration of affected lands.

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[1]Preliminary findings of this research were presented in the 1995 Wildland Shrub Symposium.

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Remedial technologies that are both environmentally beneficial and economically viable are needed. A potential method to control expansion of invading shrubs is by manipulating dietary preferences of domestic herbivores to increase selective pressures on shrubs while reducing pressures on grasses and associated grassland species. Chemical deterrents of herbivory in desert shrubs are primarily terpenoids and phenolics (Meyer and Karasov, 1991). Monoterpene content and/or profile have been related to dietary preferences of various mammals (Schwartz et al., 1980; Reichardt et al., 1985; Yabann et al., 1985; Elliott and Loudon, 1987; Bucyanayandi et al., 1990; Zhang and States, 1991). A clearer understanding of the role of these compounds in mediating interactions of grazing animals and desert shrubs should yield useful information for manipulating dietary preferences.

Currently we are using tarbush (*Flourensia cernua* DC.) to study the role of shrub chemistry in diet selection by livestock. Tarbush is an increasingly spreading species in many productive Chihuahuan Desert sites (Buffington and Herbel, 1965) with a high production potential (Estell et al., 1996), for which a biological control mechanism is desired. Tarbush is nutrient dense and contains more nitrogen than most desert shrubs (Nelson et al., 1970; Estell et al., 1996). However, depending on season and availability of other forages, tarbush is consumed by free-ranging livestock only in limited amounts (Nelson et al., 1970; Anderson and Holechek, 1983). Differential defoliation of tarbush by livestock (cattle, sheep, and goats) in a previous study at this location (Estell et al., 1994b) was related to the concentration of epicuticular wax and two unidentified terpenes. Tarbush contains several classes of secondary compounds (Kingston et al., 1975; Dillon et al., 1976; Bohlmann and Grenz, 1977; Aregullin-Gallardo, 1985), some of which may affect diet selection. Immersing tarbush in acetone or ethanol increased consumption by lambs (Estell et al., 1994a), suggesting leaf surface secondary compounds reduced tarbush acceptability to sheep. Although relatively unpalatable, tarbush leaves can be consumed safely in moderate amounts for several weeks by domestic ruminants (Fredrickson et al., 1994; King et al., 1996). However, certain growth stages of tarbush may be acutely toxic for some mammalian herbivores [i.e., flowering stage (Mathews, 1944; Dollahite and Allen, 1975)].

Our objective was to examine the relationship of tarbush leaf surface terpene profile with defoliation by livestock. Our hypothesis was that tarbush plants defoliated to a lesser extent during the previous year would contain greater concentrations of one or more leaf surface terpenes than highly defoliated plants.

METHODS AND MATERIALS

Study Site. The study was conducted on the Jornada Experimental Range (JER) in southcentral New Mexico in an area where tarbush has increased dra-

matically. The area was excluded from livestock grazing in 1988 and exposed to light to moderate stocking with cattle during the 75 years prior to livestock exclusion. Mean annual precipitation on study sites for 1989, 1990, and 1991 was 288, 267, and 393 mm, respectively. Growing season (July, August, and September) precipitation for 1989, 1990, and 1991 was 194, 174, and 231 mm, respectively. Long-term (1915–1933) mean annual and growing season precipitation for the area was 245 and 131 mm, respectively.

Sampling Protocol. This study was conducted in conjunction with a two-year study of tarbush utilization by livestock (Anderson et al., 1991). Eight paddocks (0.6 ha) at two sites (four adjacent paddocks per site; sites approximately 1.6 km apart) were densely stocked (cattle, sheep, and goats) for six to nine days (depending on forage availability) during one of two periods (approximately two weeks apart; four randomly selected paddocks browsed each period) in each of two years (1989 and 1990). High-density stocking was accomplished using cattle, sheep, and goats in a ratio of 8:20:19 and 8:23:16 per paddock in 1989 and 1990, respectively. Twenty plants in each paddock (10 in period 1 for 1989) were randomly selected and individual plant defoliation was recorded daily (visual estimation to the nearest 5% defoliation class by an experienced observer) during the browsing interval each year. Individual plants were grouped into high (HD) or low (LD) defoliation categories each year based on daily defoliation patterns (HD: $\geq 50\%$ defoliation at period end; LD: $<50\%$ defoliation at period end).

Livestock were excluded from paddocks between 1988 and 1991 except for the short browsing interval each year (1989 and 1990). Leaf samples were collected from each plant during 1991 ($N = 154$, six plants were not sampled) in late August to coincide with the mature leaf stage when plants were browsed during 1989 and 1990. Approximately 50 g of leaves were collected from each plant by removing the current year's leader growth from the outer canopy at several locations on each plant. Intact leaders were immediately placed in plastic bags, frozen on dry ice, and stored at $-10°C$. A voucher specimen of tarbush was placed in the JER herbarium located in Las Cruces, New Mexico.

Chemical Analyses. Mature leaves (including petiole) of uniform size and appearance from the midpoint of the current year's growth were subjected to chemical analyses. Dry matter was analyzed for each plant from 10 whole leaves per duplicate. Epicuticular wax [modification of the gravimetric procedure of Mayeaux et al. (1981)] was analyzed for each plant on 10 whole leaves per duplicate by extraction with 20 ml of chloroform for 20 sec. Leaf surface mono- and sesquiterpenes were extracted and analyzed by modified procedures of Estell et al. (1994c). Five whole leaves from each plant were thawed and extracted at room temperature with 5 ml of 95% ethanol for 5 min. The extract was filtered through a glass-fiber filter and refrigerated. 2-Carene (10 ng/μl) was added as an internal standard. Extracts were subjected to gas chromatography–ion trap mass spectrometry (electron impact ionization source, DB-5, 5% phenyl, 95%

methyl silicone, 30 m, 0.32 mm ID, 0.25-μm film thickness, helium as carrier gas at 1 ml/min, 300-sec filament multiplier delay time, 220°C injector temperature, 260°C transfer line temperature, initial column temperature of 50°C, 1°C/min ramp to 60°C, 3°C/min ramp to final column temperature of 240°C, 5 min isothermal, 75 min total run time, 1 μl injection volume). Tentative identification of leaf surface terpenes was based on comparison of unidentified peak spectra to the spectral library assembled by Adams (1989). Positive identification of compounds was based on comparison of retention times and spectra to those of standards. Concentrations of unidentified peaks were estimated using peak area ratios relative to the internal standard.

Statistical Analyses. All analyses relating plant chemistry to the extent of defoliation were conducted using 1990 defoliation categories because chemistry data were available for all 160 plants. Relationships of concentration of dry matter, ash, epicuticular wax, mono- and sesquiterpenes, and unidentified compounds with plant defoliation categories were examined individually (univariate analysis) using analysis of variance (MANOVA procedures) (SAS, 1989).

A variable selection procedure (M. Mahrt and D. W. Smith, personal communication) was used to select a subset of the 26 known variables (dry matter, ash, wax, identified terpenes) to subject to multivariate analysis. This procedure examines all possible subsets of variables and determines the best set for predicting membership in the two categories. The generalized squared distance function (D^2; distance between centroids of categories) is used to measure the distance between the two categories. A variable set providing maximum D^2 was determined for each number of variables, i.e., all possible combinations of 1–26 variables were examined. Maximum D^2 achieved between categories gradually decreased as the number of variables was reduced. This selection procedure identifies the subset of variables that provides the greatest D^2 for a given number of variables; however, selecting a set of variables of an appropriate size to subject to multivariate analysis is somewhat subjective. Our goal was to minimize the number of variables used to discriminate between the two defoliation categories without eliminating important variables or compromising the ability to distinguish between categories with a reasonable level of confidence. The 14-variable subset was selected for multivariate analysis because eliminating 12 variables reduced D^2 by less than 10% compared to inclusion of all 26 variables. This procedure was repeated to determine the best possible combination of the 24 unidentified variables. Removal of 10 variables resulted in a subset of 14 unidentified variables that reduced D^2 by less than 10% from the original 24 variables. The procedure was conducted separately for the known and unknown compound groups because of the complexity of the data set. These sets that distinguished HD from LD plants were subjected to multivariate analysis of variance (knowns and unknowns separately) in the MANOVA procedure of SAS (1989). The Wilks' lambda test statistic was used to test for differences between categories ($P < 0.05$).

RESULTS

Effective precipitation in 1989 and 1990 was similar to the long-term mean, while 1991 precipitation was above average. Much of the 1991 precipitation occurred in December, after samples were collected. Conditions were generally dry during the first several weeks of the growing season in both 1990 and 1991. Insect damage (primarily due to *Zygograma tortuosa* Rogers) was extensive in 1990, but was not evaluated in 1989. Insect damage was prevalent in 1991, with many shrubs exhibiting severe defoliation. The number of plants classified as HD and LD was 92 and 28 in 1989 and 132 and 28 in 1990. Of the 120 shrubs evaluated both years, 82 plants were HD both years, 13 were LD both years, 15 plants shifted from LD in 1989 to HD in 1990, and 10 plants shifted from HD in 1989 to LD in 1990.

Least square means for dry matter, ash, epicuticular wax, and individual compound concentrations are presented in Table 1. When individual compounds were subjected to univariate analysis, several leaf surface components were related to degree of herbivory the previous year (Table 1). α-Pinene and flourensadiol concentrations were greater and *cis*-jasmone concentration was lower in LD plants ($P < 0.05$). Dry matter tended to be greater and ash and *p*-cymene concentrations tended to be lower in LD plants ($P < 0.10$). The concentration of unknown 23 was greater and unknowns 2 and 12 were lower in LD plants ($P < 0.05$). Unknowns 22 and 24 tended to be lower in LD plants ($P < 0.10$).

The set of identified compounds in plants in 1991 that best distinguished between HD and LD categories in 1990 contained 14 variables (dry matter, ash, α-pinene, sabinene, 3-carene, *p*-cymene, limonene, camphor, borneol, *cis*-jasmone, β-caryophyllene, α-humulene, ledene, and flourensadiol concentrations). The set of unidentified compounds distinguishing between categories contained 14 variables (estimated concentrations of unknowns 2, 4, 7, 9, 12, 13, 15, 16, 17, 18, 19, 22, 23, and 24). Separation of the two categories was possible using the above set of known ($P < 0.001$) or unknown ($P < 0.0019$) compounds.

DISCUSSION

Results of the univariate analysis indicated dry matter and ash content, two hydrocarbon monoterpenes (α-pinene and *p*-cymene), a green leaf volatile (*cis*-jasmone), and an oxygenated sesquiterpene (flourensadiol) were related to defoliation categories. Variables identified for inclusion into the set subjected to multivariate analysis included dry matter and ash concentration, five hydrocarbon monoterpenes (α-pinene, sabinene, 3-carene, *p*-cymene, and limonene), two oxygenated monoterpenes (camphor and borneol), *cis*-jasmone, three hydrocarbon sesquiterpenes (α-humulene, ledene, and β-caryophyllene), and one oxygenated sesquiterpene (flourensadiol).

TABLE 1. TARBUSH LEAF SURFACE CHEMISTRY AND RELATIONSHIP TO 1990 DEFOLIATION CATEGORIES BASED ON UNIVARIATE ANALYSIS[a]

Variable	RT[b]	HD[b]	LD[b]
Dry matter (DM), (%)		66.3 (1.2)*[c]	71.6 (2.6)†
Ash (% of DM)		11.3 (0.1)*	10.8 (0.2)†
Epicuticular wax (% of DM)		8.3 (0.3)	8.5 (0.6)
Individual compounds (μg/g DM)			
α-Pinene	621	113.5 (14.3)‡	214.3 (30.2)§
Camphene	669	63.9 (5.5)	71.9 (11.7)
Sabinene	748	10.7 (1.0)	13.3 (2.0)
β-Pinene	764	12.9 (1.7)	18.3 (3.5)
Myrcene	810	28.0 (2.0)	28.6 (4.2)
3-Carene	868	24.2 (2.3)	25.2 (4.8)
m-Cymene	907	0.52 (0.07)	0.65 (0.16)
p-Cymene	928	5.3 (0.4)*	3.7 (0.9)†
Limonene	937	90.5 (6.9)	97.3 (14.6)
1,8-Cineole	950	24.9 (4.1)	20.2 (8.6)
Camphor	1331	3.3 (0.4)	4.5 (0.8)
Borneol	1412	222.7 (25.4)	283.8 (53.8)
cis-Jasmone	2063	46.9 (3.7)‡	26.0 (7.8)§
α-Copaene	2016	4.4 (0.5)	4.9 (1.0)
α-Gurjunene	2100	0.20 (0.03)	0.16 (0.07)
β-Caryophyllene	2133	86.4 (6.8)	76.9 (14.5)
Calarene	2162	1.4 (0.1)	1.3 (0.3)
α-Humulene	2228	18.1 (1.3)	16.5 (2.7)
Ledene	2322	0.42 (0.07)	0.40 (0.15)
trans-Nerolidol	2505	0.49 (0.06)	0.35 (0.12)
Caryophyllene oxide	2549	43.2 (3.4)	37.3 (7.1)
Flourensadiol	3196	2520.6 (175.5)‡	3431.5 (372.3)§
Unknown 1[d]	1110	714.1 (58.5)	700.1 (124.0)
Unknown 2	2292	253.1 (22.1)‡	133.6 (46.9)§
Unknown 3	2355	92.0 (13.7)	60.6 (29.0)
Unknown 4	2577	234.0 (80.4)	338.6 (170.5)
Unknown 5	2676	157.6 (22.1)	124.7 (46.8)
Unknown 6	2720	91.6 (13.4)	71.8 (28.5)
Unknown 7	2729	73.6 (11.2)	91.9 (23.7)
Unknown 8	2923	148.3 (44.8)	74.6 (95.0)
Unknown 9	3086	147.5 (21.1)	93.3 (44.7)
Unknown 10	3198	2158.2 (328.1)	1855.0 (696.0)
Unknown 11	3356	105.1 (19.1)	137.7 (40.6)
Unknown 12	3474	196.8 (21.6)‡	68.0 (45.7)§
Unknown 13	3527	137.0 (21.3)	92.7 (45.3)
Unknown 14	3581	667.1 (90.4)	770.8 (191.8)
Unknown 15	3777	0.38 (0.19)	0.0 (0.41)
Unknown 16	3844	23.5 (5.4)	17.7 (11.4)
Unknown 17	3936	1603.0 (199.0)	1267.0 (422.1)
Unknown 18	3993	1084.9 (164.7)	1269.5 (349.3)

TABLE 1. Continued

Variable	RT[b]	HD[b]	LD[b]
Unknown 19	4040	3.0 (1.3)	0.39 (2.8)
Unknown 20	4048	177.7 (36.0)	161.8 (76.4)
Unknown 21	4120	193.7 (22.6)	187.3 (48.0)
Unknown 22	4147	199.7 (29.1)*	80.1 (61.8)†
Unknown 23	4386	139.4 (19.9)‡	272.6 (42.3)§
Unknown 24	4444	35.9 (7.8)*	4.8 (16.5)†

[a] Least square means (standard error) of chemical concentrations in 1991; $N = 126$ and 28 for HD and LD categories, respectively.
[b] RT = retention time (sec); HD and LD = high and low defoliation categories, respectively.
[c] *,†Defoliation categories with different superscripts differ ($P < 0.10$). ‡,§ Defoliation categories with different superscripts differ ($P < 0.05$).
[d] Tentatively identified as artemisia alcohol.

Several compounds that were significant univariates or in the variable set subjected to multivariate analysis in our study have been related to diet selection in other systems. Elliott and Loudon (1987) found red deer rejected a pelleted diet when exposed to the odor of five monoterpenes, including α-pinene, limonene, and borneol. Zhang and States (1991) reported the concentration of sabinene was greater in ponderosa pines avoided by Abert squirrels. Limonene concentration was greater in the bark of conifer species without meadow vole damage (Bucyanayandi et al., 1990). Sinclair et al. (1988) reported camphor extracted from white spruce was a feeding deterrent for snowshoe hares when added to rabbit chow. Personius et al. (1987) indicated that *p*-cymene and camphor were related to mule deer selection among and within sagebrush taxa. Riddle et al. (1996) identified specific monoterpenes correlated either positively (cymene and camphor) or negatively (α-pinene, sabinene plus β-pinene, myrcene, limonene) with juniper intake by goats.

In contrast, compounds not important in our study have been related to herbivory in other species. Reichardt et al. (1985) reported that cineole negatively affected feeding preference of hares, and Snyder (1992) reported that ponderosa pine trees selected by Abert squirrels contained greater concentrations of β-pinene in the xylem oleoresin. Myrcene concentration was negatively related to conifer use by Abert squirrels (Zhang and States, 1991) and meadow voles (Bucyanayandi et al., 1990). Nolte et al. (1994) reported no effect on feed preference when guinea pigs were fed pellets containing limonene. We are not aware of data regarding relationships of 3-carene, *cis*-jasmone, α-humulene, ledene, β-caryophyllene, or flourensadiol with herbivory in mammals.

In a previous study at this location, dry matter, ash, and epicuticular wax concentrations were negatively related to amount of defoliation by livestock (Estell et al., 1994b). A positive relationship between water content and defoliation was also observed in the present study, and dry matter was in the set of variables used to separate the two categories. A positive relationship between plant water content and grazing preference was described earlier by Archibald et al. (1943). Components of ash such as sodium can have positive or negative influences on animal preferences, depending on the postingestive consequences of previous dietary choices (Grovum and Chapman, 1988). However, ash content was negatively related to herbivory in the previous study and positively related in this study (based on univariate analysis). Ash was also in the set of variables subjected to multivariate analysis. Methodological differences or environmental factors may explain differences between studies.

The fact that epicuticular wax was negatively related to the degree of defoliation in the previous study and unrelated to defoliation category in this study with univariate analysis was somewhat surprising. However, the fact that epicuticular wax was not in the subset of variables subjected to multivariate analysis was expected, because the analysis takes into account the interrelationships among variables, and epicuticular wax contains many of the compounds measured. Several compounds in the variable set were not significant univariate variables, probably because of inherent differences between statistical analyses (only one variable is considered at a time during univariate analysis while multivariate analysis considers all variables and their interrelationships simultaneously). We recognize that a large type I error is an unavoidable consequence of the univariate statistical analyses because of the number of tests performed. Multivariate analyses were subsequently conducted in part to address these concerns. Our goal was to identify chemicals exhibiting possible relationships with intensity of defoliation, and further bioassay studies will be conducted to evaluate promising variables.

The number of variables related to extent of defoliation with multivariate analyses illustrates the complexity of relationships among plant chemicals driving plant–animal interactions and the likelihood of synergistic and antagonistic relationships among phytochemicals involved in diet selection. We examined volatile leaf surface compounds because tarbush has a resinous exudate containing several volatile terpenes present at the plant–animal interface and because a relationship of epicuticular wax and diet selection was observed previously (Estell et al., 1994b). Other constituents (internal leaf chemicals, other classes of secondary compounds, and concentration of various nutrients) undoubtedly affect diet selection as well, which further increases the complexity of the issue.

During the browsing study, rate and extent of individual tarbush defoliation were highly variable. Because visual estimates of defoliation for individual plants were recorded daily during that study, leaf samples were not collected from

these plants during 1989 and 1990. By exploring relationships of tarbush chemistry in 1991 with defoliation of the same plants in the previous year, we assumed that heavy browsing during 1989 and 1990 did not induce plant secondary chemistry changes that persisted in 1991. Tarbush exists in a high-light, low-nutrient environment, which would be expected to limit growth to a greater extent than photosynthesis and favor accumulation of carbon-based secondary chemicals (Bryant et al., 1985). The high carbon–nitrogen ratio typical of resource-poor desert environments should favor chemical defense rather than biomass production, and slow growth rates should favor constitutive rather than induced defense (Bryant et al., 1983; Coley et al., 1985). Furthermore, mono- and sesquiterpenes are under strong genetic control relative to environmental influences in some plant species (Kainulainen et al., 1992; Michelozzi et al., 1995) and are generally less responsive to environmental influences than many phytochemicals, particularly in leaf tissue (Gershenzon and Croteau, 1991).

Although not analyzed statistically, the individual plant defoliation category was generally consistent across years. In fact, 79.2% of the plants had the same classification in 1989 and 1990 and 12.5% changed from LD to HD, while 8.3% changed from HD to LD. The fact that only 10 of 120 plants were HD in 1989 (no previous browsing) and LD in 1990 (after forced heavy browsing in 1989) suggests that chemical induction was minimal and/or short term or that differences in plant chemistry between HD and LD plants were great enough that differences existed in spite of induction. Category shifts may also reflect slight differences in patterns of tarbush consumption resulting from year to year differences in availability of other plant species.

Long-term induction responses to herbvivory cannot be ruled out, particularly given the prevalence of insect damage. Induction and release of terpenes and other volatile compounds by agronomic crops in response to insect herbivory have been demonstrated (Turlings and Tumlinson, 1992; Dicke et al., 1993; Loughrin et al., 1995). Bryant and Raffa (1995) suggested that for woody plant species, induction is a more important defense mechanism against insects, while constitutive defense is more important for mammals. Biotic stresses such as herbivory can rapidly induce terpene synthesis in many plant species (Carroll and Hoffman, 1980; Gershenzon and Croteau, 1991). However, because induction of secondary compounds is often short-lived (Faeth, 1992; Furstenburg and van Hoven, 1994; Gershenzon, 1994), long-term effects on herbivory may be less of a concern.

Leaf age and plant age can also affect terpene levels in plants, with terpene concentrations typically greater in young leaves than in mature leaves (Gershenzon and Croteau, 1991). Moreover, chemically defended plant species are generally most defended in the juvenile stage (Sinclair et al., 1988; Bryant et al., 1991). We attempted to minimize effects of leaf age by sampling at the same phenological stage as during previous browsing periods. Paddocks were

located in areas that have been infested with tarbush for a number of years, and plant age effects were assumed to be minimal.

In summary, leaf surface compounds of tarbush appeared to be related to diet selection in the preceding year. Variable sets containing 14 known or 14 unknown compounds were identified that distinguished between the two defoliation categories when subjected to multivariate analysis. Other secondary chemicals in tarbush leaves (e.g., phenolics) and/or nutrient composition may also influence degree of tarbush herbivory by livestock.

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URIC ACID: THE SPERM-RELEASE PHEROMONE OF THE MARINE POLYCHAETE *Platynereis dumerilii*

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Abstract—In the marine polychaete, *Platynereis dumerilii*, reproductive behavior in the two sexes is synchronized by the consecutive discharge of male and female sex-specific pheromones. After the female releases the eggs into the free water column, immediate fertilization is achieved by several males circling around the eggs emitting sperm clouds. We report the isolation and identification of the sperm-release pheromone present in the coelomic fluid of sexually mature females. Each step in isolation was guided by bioassay. Isolation methods included extraction and solvent partitioning and separation methods included ultrafiltration and high-performance liquid chromatography. Uric acid was identified as the sperm-release pheromone that is discharged by the female with release of the eggs. The threshold concentration for sperm release by males was determined as 0.6 μM.

Key Words—Sex pheromone, sperm release, egg release, uric acid, purine, reproduction, polychaete, Annelida, *Platynereis dumerilii*, coelomic fluid.

INTRODUCTION

In contrast to much experimental evidence suggesting the presence of sex pheromones synchronizing the reproduction process of marine invertebrates, only a few such substances have been chemically elucidated (Kittredge et al., 1971; Zeeck et al., 1988). Because of distinctive breeding behavior, the marine polychaete *Platynereis dumerilii* (Annelida, Polychaeta) is well suited for both biological and chemical studies of its sex pheromones. During their development to sexual maturity, individuals undergo a metamorphosis in which the gut and

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the ability to ingest food disappear. After this metamorphosis, the semelparous heteronereids leave their living tubes at midnight and assemble near the water surface during the first week after the new moon (Hauenschild, 1956). High population densities within restricted areas and synchronization in time are responsible for the formation of swarms of both males and females. Within these swarms, mate recognition is achieved by chemical signals. The compound 5-methyl-3-heptanone seems to play a role in mate recognition (Zeeck et al., 1988), but more important is an unidentified pheromone emitted only by females (M. Beckmann, T. Harder, E. Zeeck, unpublished results). The reproductive process occurs as follows. First, at the moment of mate recognition, the male discharges an egg-release pheromone (Boilly-Marer, 1974). Second, the pheromone stimulates females to swim with high velocity in narrow circles surrounded by swarming males. Third, after an induction period of 10–40 sec, the female spawns, and the discharged cloud of coelomic fluid and eggs includes the sperm-release pheromone. Fourth, upon detection of this pheromone, males circling around the egg cloud are stimulated to emit visible amounts of sperm, and fertilization of eggs in the water occurs.

Previous experiments of Boilly-Marer (1974, 1986) revealed the heteronereids body fluid to be the source of the gamete-releasing pheromones. The coelomic fluid circulates along the entire body length (Hauenschild and Fischer 1969) and is released at the moment of spawning.

In the present study we report the isolation and identification of the sperm-release pheromone from female *Platynereis dumerilii*, which was briefly summarized previously by Zeeck et al. (1996).

METHODS AND MATERIALS

Biological Organisms. Sexually mature female heteronereids of *Platynereis dumerilii* for pheromone isolation, and males for behavioral bioassays, were obtained from our laboratory culture. About 3000 specimens were maintained and bred according to the method of Hauenschild and Fischer (1969). Natural seawater (salinity 3.2%, or 32 g/10^3 g seawater) was obtained from the North Sea, filtered over charcoal, and pasteurized at 80°C for 30 min. All solvents and chemicals were of analytical grade.

Bioassay Techniques. In order to separate and study the sperm-release pheromone, a reliable bioassay as a means to follow the target substance during isolation and purification was indispensable, and the following procedures were followed.

For the qualitative bioassay, samples for assay were injected with a microliter syringe just in front of a mature male specimen swarming in a glass dish of seawater (40 ml). The spontaneous release of a visible sperm cloud repre-

sented a positive qualitative pheromone response. Whenever a male failed to release sperm (a negative response), its ability to release sperm was tested with biologically active coelomic fluid from females. Each step of pheromone separation was accompanied by examination of bioactivity.

For the quantitative bioassay, swarming males were placed in a series of glass dishes containing seawater with decreasing pheromone concentrations, and the lowest effective concentration causing sperm release was ascertained.

Sample Preparation. The pheromone-containing coelomic fluid from 700 sexually mature females was obtained by very carefully pressing the cleaned worms with tweezers. This stimulus usually caused the females to release their eggs. Centrifugation of the coelomic fluid produced a clear supernatant and a pellet mainly of eggs. The supernatant was freeze-dried and the residue subjected to solid–liquid extraction first with acetone and then with water (Milli-Q Reagent-Water-System, Millipore). Membrane ultrafiltration (Diaflo membranes, Amicon) of the aqueous extract in consecutive filtration steps (10 kDa, 1 kDa, 0.5 kDa) yielded a pheromone-containing filtrate. The sample was concentrated by rotary evaporation and kept under argon at −20°C.

Chromatographic Procedures. In order to isolate the pheromone from the aqueous filtrate, reverse-phase, high-performance liquid chromatography (HPLC) was conducted with a Kontron HPLC system (HPLC Pump 420, Gradient Former 425) equipped with a semipreparative column (LiChrospher 100 RP18, 10 μm, 250 × 10 mm; Merck). Signal detection was established with a variable wavelength UV detector (Uvicon 720 LC). The mobile phases of water (pH 6.5), water–0.1% trifluoroacetic acid (TFA) (pH 2.5), and methanol–water (60:40, v/v) were either used isocratically or as a binary gradient. Chromatographic fractions were collected, freeze-dried, dissolved in water, and finally checked in the bioassay for pheromonal activity. In order to test for the possibility of a pheromone complex, each fraction was tested both singly and in combination with the others. Biologically active fractions were chromatographed (HPLC) with different mobile phases to isolate single peaks.

Aliquots of single peak fractions were examined by thin-layer chromatography (TLC) performed on silica gel plates (60-F_{254}, 0.20 mm, Merck) with the developing solvents ethanol (96%)–water (3:1, v/v) and *n*-butanol–acetic acid (99%)–water (4:2:1, v/v/v). Spots were detected with UV light (254 nm) and with three staining reagents applied on the same plate in the order; iodine vapor, ninhydrin, and (after chlorination) *o*-tolidine. The same sample was run under identical conditions on another plate that was stained (charred) with sulfuric acid.

In order to detect the sperm-release pheromone directly in seawater immediately after egg discharge by females, gel-filtration chromatography (GFC) was performed. Samples of seawater (10 ml) containing pheromone were obtained by stimulating mature females to release eggs in the absence of males by gentle

irritation with tweezers. Eggs were separated by centrifugation. The supernatant (spawning water) was concentrated to 1 ml, desalted, and analyzed by UV absorbance. GFC was performed with a Superformance column (16 × 600 mm, Merck; package: HW40 (S) resin, ToyoHaas) connected with the HPLC system described above using water as mobile phase. Samples of concentrated natural seawater were analyzed under identical conditions in order to obtain reference data.

Analysis and Identification. UV spectroscopic analysis of biologically active, single-peak fractions was carried out with a Uvicon-810 photospectrometer. Proton magnetic resonance (^{1}H NMR) spectra were recorded at 500 MHz with a Bruker ARX500 spectrometer. Deuterium oxide was employed as solvent, and the pH was adjusted to pH 9 with sodium deuteroxide. Mass spectrometric (MS) analysis was performed with a double-focusing Finnigan MAT 95 mass spectrometer. Electron impact mass spectra (direct inlet) were obtained at 70 eV, 200–400°C. Identification of the pheromone was confirmed by comparison of the chromatographic and spectroscopic characteristics of the natural product with those of a reference compound analyzed under the same conditions, and decisive proof of identity was the equivalent biological activity of a synthesized or commercially available substance.

RESULTS

A flow chart of the bioassay-guided sample preparation is shown in Figure 1. Subsequent isolation of the sperm-release pheromone was achieved by HPLC in three optimized chromatographic steps (Figure 2): (1) An aliquot of the 0.5-kDa filtrate (10 females) was applied to the RP18 column (for separation conditions see Figure 2 legend). A pheromone-containing fraction (designated by hatched area) was obtained by elution with a binary gradient of water–(methanol–water). This fraction was freeze-dried and redissolved in water–0.1% TFA. (2) Rechromatography of the biologically active fraction of the first step on the same chromatographic system and isocratic elution with the acid mobile phase water–0.1% TFA led to a single pheromone-containing peak fraction (hatched area), which was freeze-dried and redissolved in water. (3) Rechromatography of this fraction on the same chromatographic system and isocratic elution with water revealed two peaks. The pheromonal activity was definitively detected in the second peak (Figure 2, hatched area). The chromatographic purity of this fraction was proved by variation of the mobile phases. After freeze-drying, the yield of the final product was 9 ± 1 μg from 10 females.

A characteristic UV spectrum of the biologically active single peak was obtained with $\lambda_{max\,1}$ at 234 nm, $\lambda_{max\,2}$ at 290 nm, $\lambda_{min\,1}$ at 219 nm, and $\lambda_{min\,2}$ at 260 nm. The spectrum suggested a pure substance. TLC was used to examine

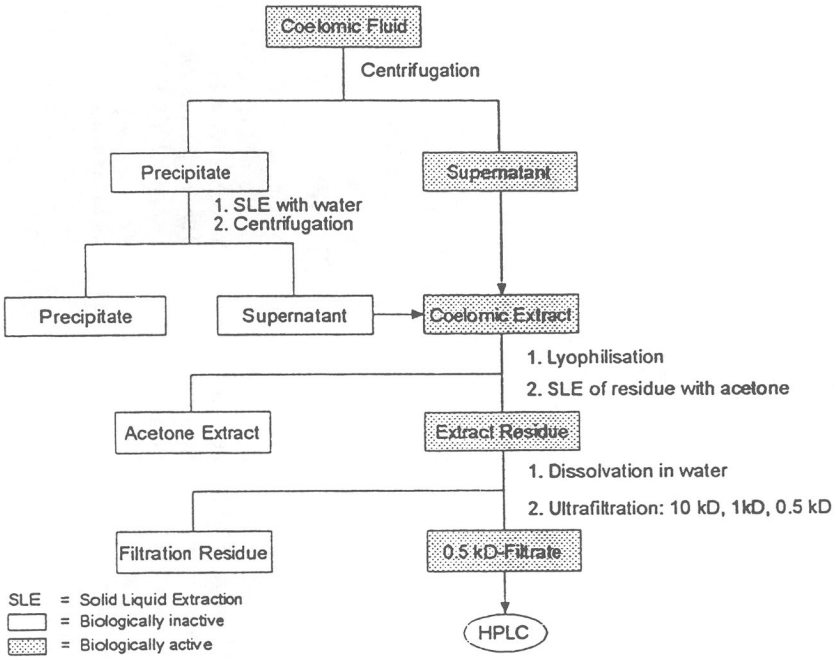


FIG. 1. Flow chart of the bioassay-guided sample preparation.

chemical purity of this sample. Development of the TLC plate with 96% ethanol–water (3 : 1) produced a single spot at retention factor (R_f) = 0.80. Development of a plate with *n*-butanol–acetic acid–water (4 : 2 : 1) gave a single spot at R_f = 0.61. Spots were stained with iodine vapor to detect any unsaturated compounds and compounds with nitrogen such as guanidine bases, pyrimidines, and purines (Brante, 1949). Sulfuric acid was used to detect oxidizable compounds in general, with *o*-tolidine to detect compounds that can be converted to chloramines (Reindel and Hoppe, 1954). Ultraviolet light was used to detect compounds that fluoresce and/or absorb UV light at 254 nm. Ninhydrin, which detects primary amines and amino acids, did not stain. Because TLC with several different solvent systems produced only one spot, the biologically active single-peak fraction obtained by HPLC constituted a pure compound.

Analysis of the UV spectrum and results of analytical microreactions on the TLC plates indicated a heteroaromatic structure with intramolecular amide bonds. Quantitative isolation of the pheromone from the coelomic fluid of 700 females yielded 0.7 mg. Despite extensive NMR spectroscopic measurements no ^1H signals were received.

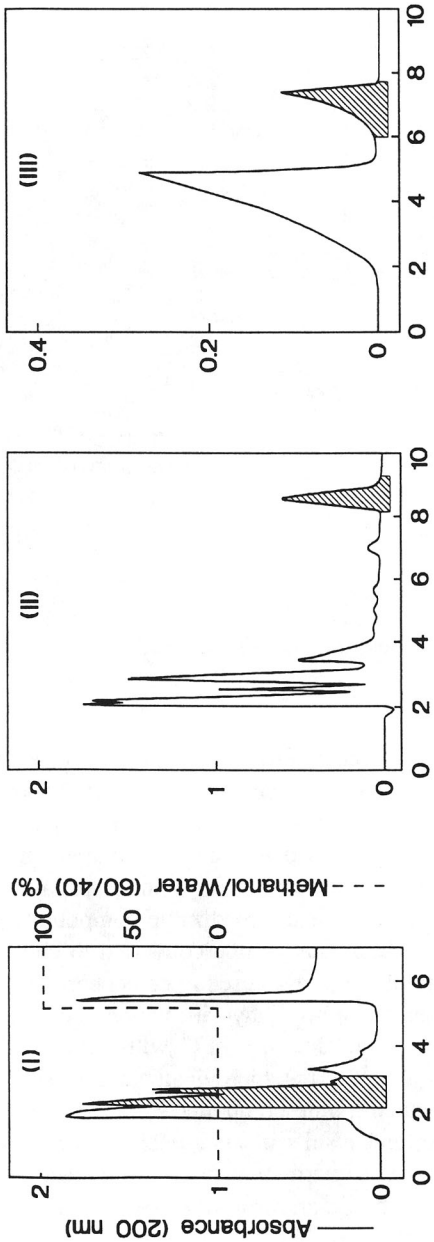


FIG. 2. HPLC isolation of the sperm-release pheromone of *Platynereis dumerilii* in three chromatographic steps. (I) Column: LiChrospher 100 RP18, 250 × 10 mm, 10 μm; mobile phase: A, water; B, methanol–water (60:40, v/v), 6 ml/min; UV detection at 200 nm. The sample was 500 μl aqueous 0.5-kDa filtrate from 10 females. (II) Column: LiChrospher 100 RP18, 250 × 10 mm, 10 μm; mobile phase: water–0.1% TFA, pH 2.5, 6 ml/min; UV detection at 200 nm. The sample was the biologically active fraction of step I above, concentrated in 100 μl water–0.1% TFA. (III) Column: LiChrospher 100 RP18, 250 × 10 mm, 10 μm; mobile phase: water, pH 6.5, 6 ml/min; UV detection at 200 nm. The sample was the biologically active fraction of step II above, concentrated in 100 μl water. The biologically active chromatographic fractions are marked by hatched areas.

The indication of a heteroaromatic structure with intramolecular amide bonds in combination with no detectable protons by NMR suggested that the purine uric acid was the pheromone. This suggestion was in accordance with the UV spectrum and the retention values of synthetic uric acid obtained by HPLC and TLC. Due to their acidic character, the four hydrogen atoms of uric acid undergo exchange with deuterium if uric acid is dissolved in deuteriumoxide. Consequently, no signals are recorded in ^1H NMR. The final proof of identity was secured by mass spectrometry. An aliquot of the pheromone was analyzed by MS with direct inlet. The spectrum showed major ions at *m/z* (relative abundance) 168 ($M^{+\cdot}$, 93.13), 125 (100), 97 (18.4), 69 (36.65), and 54 (69.11). The abundant ion fragment at *m/z* 125 is attributed to a retro-Diels-Alder reaction in accordance with xanthines and uracils (Pfleiderer, 1974). The MS data and steps of fragmentation (Figure 3) are in excellent agreement with literature data for uric acid (Lifshitz et al., 1968).

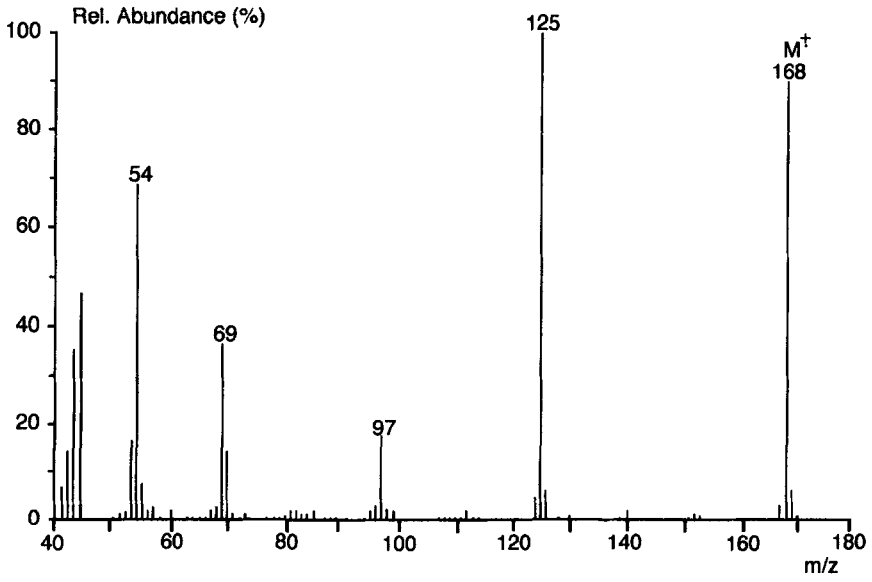
In the qualitative behavioral bioassay the biological activity of commercially available synthetic uric acid was confirmed. After addition of an aqueous solution of uric acid, mature males immediately circled around the injection spot releasing visible clouds of sperm. Quantitative bioassay indicated the threshold concentration for male response to uric acid was 0.6 μM. The maximum solubility of uric acid in seawater (3.2% salt) at pH 8.2 and 23°C was determined spectrophotometrically (from the log extinction coefficient at 291 nm = 4.09 at pH 8.0) as 180 μM, although it is generally described as very insoluble in water. The structural formula of the monoanion is given in Figure 4.

The total quantity of solubilized uric acid in the coelomic fluid of one female was isolated from spawning water by GFC and determined spectrophotometrically as 4.0 $\pm$ 0.1 μg. This method represented the easiest access to the sperm-release pheromone at a minimal loss but was unsuitable for enriching the compound due to the high salinity of the sample.

By applying the same method to concentrated samples of natural seawater, uric acid was shown to be absent in the water. Its absence in seawater is important to its function as a potential chemical signal, because the signal must not be ubiquitious in the natural habitat.

DISCUSSION

In the marine polychaete *Platynereis dumerilii* uric acid acts as a reproduction coordinating semiochemical. Uric acid is a common excretory product of many terrestrial animals, including insects and birds. The threshold concentration of uric acid for causing sperm-releasing behavior of males is 0.6 μM and appears to be high compared to other known marine pheromones, which are effective at concentrations in the picomole range (Rittschoff et al., 1985,



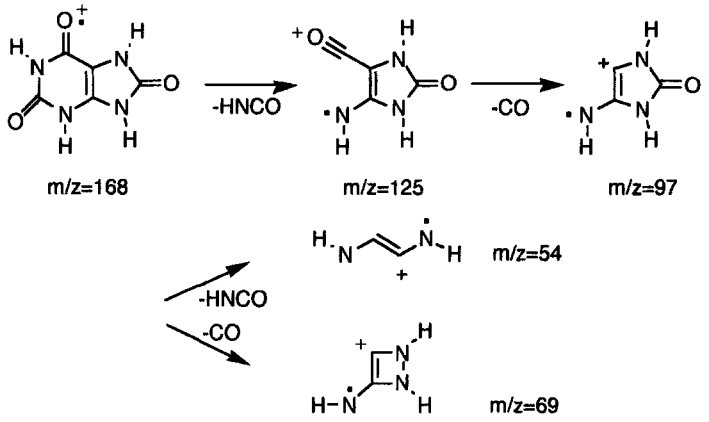


FIG. 3. Mass spectrum (EI, direct inlet, 70 eV, 220–400°C; Finnigan MAT 95) and characteristic fragmentation ions of the pheromone.

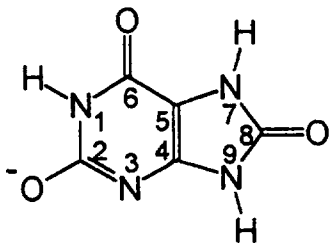


FIG. 4. Structural formula and ring nomenclature of the monoanion of uric acid.

1989; Boland et al., 1981). However, the relatively high threshold concentration may be ecologically relevant because a low threshold concentration presumably would induce the sperm releasing behavior of males not only close to the egg cloud, but also far more distant from the spawning female, possibly wasting gametes that would not find an egg to fertilize.

Calculation of the essential volume of seawater to dissolve the total uric acid released by one female (4.0 $\pm$ 0.1 μg) and produce the response threshold concentration of 0.6 μM is 40 ml of seawater. Actually, the volume of water that contains an egg cloud is approximately 15 ml (personal observation), indicating a 2.5-fold higher pheromone concentration than the threshold. This surplus seems to be essential to counteract effects of dilution caused by turbulence and currents and to ensure successful egg fertilization in the free water column of the natural habitat. Recent experiments concerning the pheromone decomposition in natural seawater showed that uric acid is oxidized to allantoin, a compound with no pheromonal activity (Harder, 1996). Thus, oxidation, as well as dilution, reduces the concentration of the pheromone in the water.

The high specificity of the pheromone receptor was proved with substances chemically closely related to uric acid. In total, 28 different compounds were checked in the bioassay for pheromonal activity (Harder, 1996). Small deviations in the structure of uric acid led to a loss of biological activity, e.g., 1-methyl-, 7-methyl-, and 9-methyl-uric acid induce no pheromone response in *Platynereis dumerilii*. The only exceptions found were 3-methyl-uric acid and xanthine, which induce the sperm-release behavior but only at a 100-fold higher threshold concentration than the natural pheromone.

We identified another purine acting as a gamete-release pheromone in the related annelid *Nereis succinea* (Zeeck et al., 1996). Males of this polychaete discharge an egg-release pheromone complex in which inosine was identified as the main component. Our results suggest both uric acid and inosine are species- and sex-specific. They represent the first identified gamete-release pheromones in marine invertebrates. The purine ring system has not previously been reported to be involved in sex pheromones. Currently, we are trying to isolate the egg-

release pheromone of *P. dumerilii*. The sperm-release pheromone of *N. succinea* is identified as L-cysteine-glutathione disulfide (Zeeck et al., 1998).

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INTER- AND INTRASPECIFIC VARIATIONS IN PRODUCTION OF SPINES AND PHENOLS IN *Prosopis caldenia* AND *Prosopis flexuosa*

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Abstract—In central Argentina the leguminous tree *Prosopis caldenia* is more abundant in sites of relatively high productivity (lowlands), whereas *P. flexuosa* is more abundant in sites of relatively low productivity (slopes and uplands). Based upon current antiherbivore defense theory, we predicted: (1) a higher investment in defenses in *P. flexuosa* than in *P. caldenia*, and (2) that limitations in resources would result in an increase of the defenses in both species. Our approach for testing these predictions was to estimate leaf phenol concentration and spinescence in adults (field study) and seedlings (greenhouse study) of both species growing at different levels of resource availability. In adult plants, the concentration of phenols was higher ($P < 0.01$) in *P. flexuosa* than in *P. caldenia*, but the opposite relationship was observed in seedlings. The amount of biomass invested in spines was similar ($P > 0.10$) in both species, whereas spine density was higher ($P < 0.05$) in *P. caldenia* than in *P. flexuosa*. In both species, limitations in resources did not result ($P > 0.05$) in increases in leaf phenol concentration, amount of biomass invested in spines, spine length, or spine density, except for the increase ($P < 0.05$) of spine density in seedlings of *P. caldenia* at low water and nutrient availability. In general, our results did not support current hypotheses on the production of antiherbivore defenses. It is argued that factors such as herbivore behavior (e.g., habitat selection, trampling, branch breakage) and alternative sinks for carbon (e.g., N_2 fixation, carbohydrate reserves), in addition to resource availability in evolutionary and ecological time, should be considered for a more complete understanding of the inter- and intraspecific variations in the production of both physical and chemical antiherbivore defenses.

Key Words—Antiherbivore defenses, physical defenses, chemical defenses, phenols, spines, *Prosopis caldenia*, *Prosopis flexuosa*, Argentina.

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INTRODUCTION

In the last two decades, considerable efforts have been devoted to understanding inter- and intraspecific variations in the production of antiherbivore defenses. To explain interspecific variations, ideas have shifted from a focus on plant apparency (Feeny, 1976; Rhoades and Cates, 1976) to resource availability (Bryant et al., 1983; Coley et al., 1985). The resource availability hypothesis suggests that the level of defense increases as the potential growth rate of the plant decreases. The rationale is that species adapted to resource-rich habitats can compensate for relatively high levels of herbivory by rapid growth and tissue replacement. In contrast, species adapted to resource-poor habitats where growth is restricted must invest more in defenses to evade herbivory. On the other hand, two hypotheses have been proposed to explain intraspecific variations in antiherbivore defenses in response to resource limitation. The carbon–nutrient balance hypothesis (Bryant et al., 1983) argues that under conditions of nutrient limitation, plants accumulate large amounts of carbohydrate reserves that are used in the production of carbon-based defenses, whereas under conditions of carbon limitation (e.g., due to insufficient light) carbon-based defenses decrease. The carbon–nutrient balance hypothesis is a component of the growth-differentiation balance hypothesis (Herms and Mattson, 1992), which suggests that under any condition of resource limitation that slows growth, plants accumulate carbohydrate reserves that are used in the production of carbon-based defenses. The fundamental premises of the last two hypotheses are the existence of a physiological trade-off between growth and production of defenses and that under conditions of resource limitation, growth is limited to a greater extent than photosynthesis. However, neither the carbon–nutrient balance hypothesis nor the growth–differentiation balance hypothesis take into account alternative competing sinks for carbon, such as storage and nitrogen fixation in N_2-fixing species.

Prosopis caldenia and *P. flexuosa* are leguminous trees that grow in the central semiarid region of Argentina, although *P. flexuosa* is also able to grow in more arid zones. Within the semiarid region, *P. caldenia* is more abundant in sites of relatively high productivity (lowlands), whereas *P. flexuosa* is more abundant in sites of relatively low productivity (slopes and uplands) (Cano, 1988; Bóo and Peláez, 1991). Based upon current antiherbivore defense hypotheses, we predicted a higher investment in defenses in *P. flexuosa* (from sites of relatively low productivity) than in *P. caldenia* (from sites of relatively high productivity) and that limitations in resources would increase the level of defenses in both species. Our approach for testing these predictions was to estimate leaf phenol concentration and spinescence (biomass invested in spines, spine density, and spine length) in adults and seedlings of both species growing at different levels of resource availability.

METHODS AND MATERIALS

Study Site and Species. The field study was conducted in the Caldén District (Cabrera, 1976) on a site located in the southeastern zone of La Pampa province (38°45′S; 63°45′W). The climate of the region is temperate and semiarid. Mean monthly temperatures range from 7°C in July to 24°C in January, with an annual mean of 15°C. Mean annual rainfall is 400 mm, with peaks in fall and spring. The more severe droughts occur during summer. Precipitation during the study period was 393, 396, 363, and 267 mm in 1992, 1993, 1994, and 1995, respectively. Dominant soils are classified as Calciustolls of medium to coarse texture. Soils are distributed in three topographic positions: lowlands, uplands, and slopes. In uplands, soils are quite shallow with a petrocalcic layer at around 50 cm in depth, whereas in lowlands and slopes, the petrocalcic layer is commonly below 150 cm in depth. The biggest trees of *P. caldenia* and *P. flexuosa* grow on lowlands, indicating that in these sites conditions are less limiting for growth than those on slopes and uplands. The physiognomy of the vegetation is grassland with scattered trees or shrubs on uplands, shrubland on slopes, and open woodland on lowlands. Woody species are mainly represented by *P. caldenia*, *P. flexuosa*, *Larrea divaricata*, and *Condalia microphilla*, whereas herbaceous species are represented by several species of the genus *Stipa*, *Piptochaetium napostaense*, *Poa ligularis*, *Medicago minima*, and *Erodium cicutarium*.

P. caldenia and *P. flexuosa* are warm-season, caducifolius, leguminous trees, native to Argentina (Burkart, 1976). In the study site, they normally start producing leaves in October and lose their leaves in April (Distel and Peláez, 1985). Both species produce a pair of straight, long spines at each axillary bud. There is almost no information on their secondary chemistry. Presently, the studied species are subjected to low herbivory from both wild and domestic animals. However, goats browse on *P. caldenia* in summer (M. Pisani, personal observation), and they preferred this species over *P. flexuosa* in a preference trial (Pisani et al., 1995).

Field Study. Adult plants of *P. caldenia* and of *P. flexuosa* ($N = 20$ in 1993, and $N = 10$ in 1995) were randomly selected in both an upland site and a lowland site. Plants of both species were the same size (2–3 m high) and were growing on similar soil in each site. These plants were at the beginning of their reproductive life and produced only a few pods during the study period. In the first week of April 1993 and 1995, four branches of the same basal diameter (8 mm) and ramification order were harvested from each individual tree. Samples were air dried and weighed. The low drying temperature minimized denaturing of carbon-based secondary metabolites (Hagerman, 1988). The biomass of current-season twigs was weighed separately to estimate twig production in *P. caldenia* and *P. flexuosa*. Finally, the biomass was sorted into leaves and stems. Leaves were milled to pass a 1-mm sieve and analyzed for total phenols

following the method of Price and Butler (1977). Phenolic compounds were quantified because legumes trees are particularly rich in this class of natural products (Janzen, 1981; Lyon et al., 1988). Total phenols were extracted from 60 mg of leaf powder with 3 ml of methanol for 15 hr at room temperature. The extract was filtered, the extraction tube rinsed with an additional 3 ml of methanol, and the filtrate added to 50 ml of distilled water. Then, 3 ml of 0.1 M $FeCl_3$ in 0.1 N HCl was added to the extract. Exactly 20 min after the addition of the ferric chloride, 3 ml of 0.008 M $K_3Fe(CN)_6$ was added. Finally, exactly 20 min after the addition of the ferricyanide, an aliquot of 1.5 ml of methanolic extract was diluted by the addition of 1.5 ml of distilled water and the absorbance read in a 1-cm glass cuvette at 720 nm. A blank of identical composition, but omitting the leaf extract, was analyzed and subtracted from all other readings. Phenol concentrations were expressed as absorbances, as recommended by Martin and Martin (1982). Stems were used to determine biomass invested in spine (milligrams of spine per gram of branch), spine density (number of spines per centimeter of branch), and spine length (millimeters). These spinescence variables were estimated in 1993 only.

Greenhouse Study. In November 1993, seedlings of *P. caldenia* and *P. flexuosa* obtained from seeds collected from the study site were transplanted into 16-cm × 50-cm-deep pots filled with soil from the same site (9.7 kg soil per pot: 1.51% organic matter; 0.12% total nitrogen; 9.44 ppm extractable phosphorus; 458 ppm potassium; 7.97 pH). After transplantation, all pots were kept well watered for one month to favor seedling establishment. The final seedling density was two per pot. The seedlings of both species were subjected to four treatments resulting from the combination of two levels of water availability and two levels of fertilization. In the high (wet) and low (dry) water treatments, the water content of the soil was kept at 15% (soil field capacity) and 7.5% (half soil field capacity), respectively, by weighing the pots twice weekly and supplying the required amount of water. The levels of fertilization were either not fertilized or fertilized by the addition of 145 ml of a nutrient solution twice a week, coincident with irrigation. The 145 ml of the nutrient solution provided 81.8 mg N, 103 mg K, and 8.1 mg P. Each treatment was replicated 10 times. The experimental unit was a pot with two seedlings. The experiment was terminated after 18 weeks, when the above- and belowground biomass were harvested. The belowground biomass was obtained by carefully washing the soil from the roots. The biomass was dried at 40°C to a constant weight and weighed. The results were expressed as grams per pot. Total phenols and spinescence variables were measured following the procedures described for the field study.

Statistical Analysis of Data. The data were analyzed by either a two-way ANOVA (field study: two species × two sites) or a three-way ANOVA (green-

house study: two species × two levels of water × two levels of fertilization). When the variance was heterogeneous, the data were either ln-transformed (total biomass and root biomass of seedlings) or square root-transformed (root–shoot ratio of seedlings) prior to analysis. However, the nontransformed variables are shown in tables and figures. The data on production of current season twigs of adult plants (field study) were analyzed by the Kruskal-Wallis nonparametric test.

RESULTS

Field Study. The production of current season twigs (Table 1) was not affected ($P > 0.10$) by species, except for the higher ($P < 0.05$) twig production of *P. flexuosa* in the upland site in 1993, and it was affected ($P < 0.01$) by site in 1993 only. In 1993, twig production in the lowland site was higher than in the upland site. The lack of difference in twig production between the upland and lowland site in 1995 was associated with below average precipitation in 1994, which can be inferred to have reduced the recharge of water of deep soil layers in lowlands. However, in December 1994 precipitation was above average (125 mm) and promoted a relatively high twig production in both the upland site and the lowland site.

The concentration of total phenols in leaves was affected ($P < 0.01$) by species and site in both years (Table 2). The interaction species × site was significant ($P < 0.05$) in 1993 only. The concentration of phenols was higher in *P. flexuosa* than in *P. caldenia* both in 1993 and 1995, and it was higher in the lowland site than in the upland site in 1993, but the opposite happened in 1995. The treatment interaction computed in 1993 resulted from the similar

TABLE 1. CURRENT SEASON TWIG PRODUCTION OF ADULT PLANTS OF *Prosopis caldenia* AND *Prosopis flexuosa*[a]

	Production g/branch			
	1993		1995	
Site	*P. caldenia*	*P. flexuosa*	*P. caldenia*	*P. flexuosa*
Upland	0.2 ± 0.1 a	0.9 ± 0.4 b	2.4 ± 0.4 a	2.6 ± 0.7 a
Lowland	3.1 ± 0.5 c	3.2 ± 0.4 c	2.2 ± 0.4 a	1.2 ± 0.4 a

[a]Values are mean ± 1 SE ($N = 20$, 1993; $N = 10$, 1995). Different letters within years indicate significant ($P < 0.05$) differences according the Kruskal-Wallis nonparametric test.

TABLE 2. FOLIAR CONTENT OF TOTAL PHENOLS IN ADULT PLANTS OF *Prosopis caldenia* and *Prosopis flexuosa*[a]

	A_{720}/60 mg dry weight			
	1993		1995	
Site	*P. caldenia*	*P. flexuosa*	*P. caldenia*	*P. flexuosa*
Upland	1.26 ± 0.03 a	1.38 ± 0.02 b	1.21 ± 0.08 a	1.44 ± 0.09 a
Lowland	1.39 ± 0.03 b	1.41 ± 0.02 b	0.83 ± 0.09 b	1.22 ± 0.08 a

[a] Values are mean ± 1 SE (N = 20, 1993; N = 10, 1995). Different letters within years indicate significant ($P < 0.05$) differences (LSD test) (Snedecor and Cochran, 1980). The content of total phenols was affected ($P < 0.01$) by species and site in both years. The interaction species by site was significant ($P < 0.05$) in 1993 only.

concentration of phenols in plants of *P. flexuosa* from the upland and lowland sites.

The amount of biomass invested in spines was not affected ($P > 0.10$) either by species or site, whereas spine density and length were affected ($P < 0.05$) by species only (Table 3). There was no species × site interaction ($P > 0.10$). Both the density and length of spines were higher in the branches of *P. caldenia* than in the branches of *P. flexuosa*.

Greenhouse Study. The total biomass production of seedlings was affected ($P < 0.01$) by species, water level, and fertilization level (Figure 1). There were no interactions ($P > 0.1$) among treatments. Seedlings of *P. caldenia* grew more rapidly than did seedlings of *P. flexuosa*, and the higher the level of water and the level of fertilization, the faster the growth of the seedlings of both species. Root–shoot ratio was affected ($P < 0.01$) by species, water level, and fertilization level (Figure 2). Seedlings of *P. caldenia* had a higher root–shoot ratio than seedlings of *P. flexuosa*, and the lower the level of water and of fertilization, the higher the root–shoot ratio.

The concentration of total phenols in leaves was affected ($P < 0.01$) by species only (Figure 3). There were no interactions ($P > 0.10$) among treatments. Phenol concentrations were higher in *P. caldenia* than in *P. flexuosa*.

The amount of biomass invested in spines was not affected ($P > 0.05$) either by species, water level, or fertilization level (Figure 4). Spine density was affected by species ($P < 0.01$), water level ($P < 0.05$), and fertilization level ($P < 0.01$) (Figure 5). The interaction species × water level was significant ($P < 0.01$). Spine density was higher in *P. caldenia* than in *P. flexuosa*, and the higher the level of water and fertilization, the lower the spine density.

TABLE 3. BIOMASS INVESTED IN SPINES, DENSITY, AND LENGTH OF SPINES IN ADULT PLANTS OF *Prosopis caldenia* AND *Prosopis flexuosa*[a]

Site	Biomass Invested (mg spines/g branch)		Density (spines/cm branch)		Length (mm)	
	P. caldenia	*P. flexuosa*	*P. caldenia*	*P. flexuosa*	*P. caldenia*	*P. flexuosa*
Upland	39.1 ± 6.2 a	32.7 ± 7.2 a	0.64 ± 0.03 a	0.36 ± 0.02 b	11.5 ± 0.97 a	10.2 ± 0.88 ab
Lowland	33.6 ± 4.3 a	31.1 ± 8.1 a	0.62 ± 0.03 a	0.38 ± 0.03 b	11.1 ± 0.83 ab	8.5 ± 1.03 b

[a]Values are mean ± 1 SE ($N = 20$). Different letters within variables indicate significant ($P < 0.05$) differences (LSD test) (Snedecor and Cochran, 1980). The amount of biomass invested in spines was not affected ($P > 0.10$) either by species or site. Spine density and length were affected ($P < 0.05$) by species only. For both variables, the interaction species by site was not significant ($P > 0.10$).

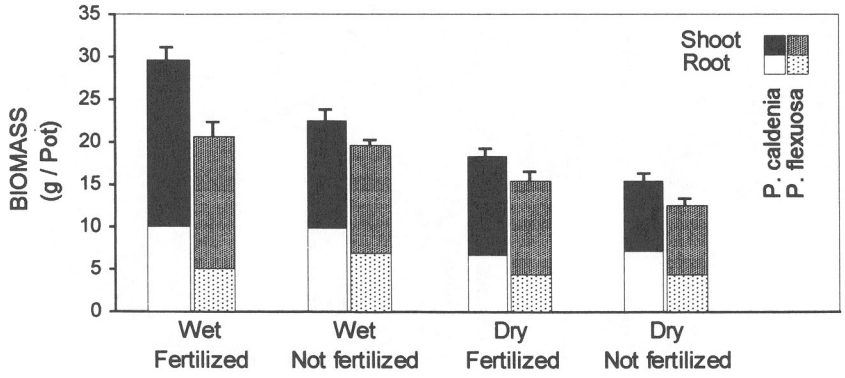

FIG. 1. Total biomass production of seedlings of *Prosopis caldenia* and *Prosopis flexuosa* in the greenhouse study. Values are mean for 10 replicates. Error bars show SE for total biomass production. Total biomass production was affected ($P < 0.01$) by species, water level, and fertilization level. There were no interactions ($P > 0.10$) among treatments.

However, the species × water level interaction resulted from similar spine density of *P. flexuosa* seedlings, regardless of the water level. The length of spines was not affected ($P > 0.10$) either by species, water level or fertilization level (Figure 6).

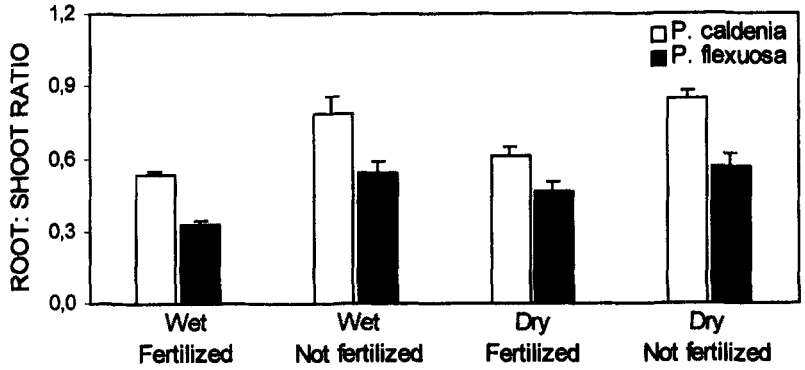

FIG. 2. Root-shoot biomass ratio of seedlings of *Prosopis caldenia* and *Prosopis flexuosa* in the greenhouse study. Values are mean ± 1 SE ($N = 10$). Root-shoot biomass ratio was affected ($P < 0.01$) by species, water level, and fertilization level. There were no interactions ($P > 0.10$) among treatments.

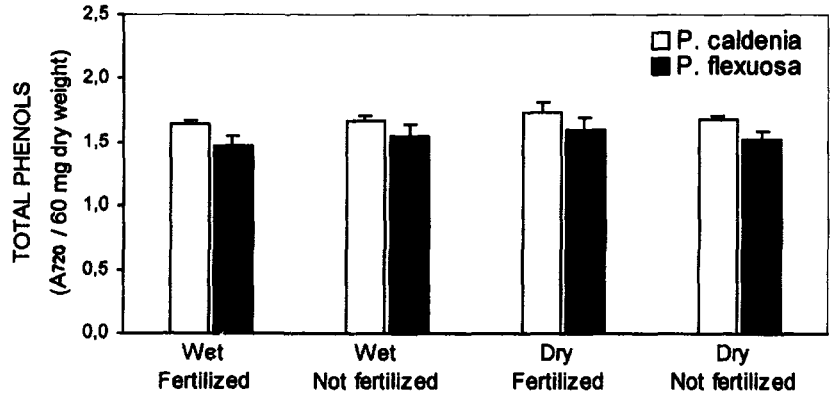

FIG. 3. Foliar content of total phenols in seedlings of *Prosopis caldenia* and *Prosopis flexuosa* in the greenhouse study. Values are mean ± 1 SE ($N = 10$). The content of total phenols was affected ($P < 0.01$) by species only. There were no interactions ($P > 0.10$) among treatments.

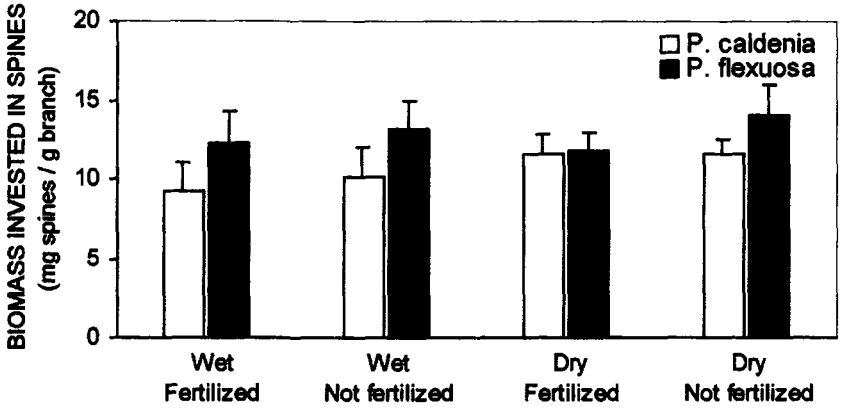

FIG. 4. Biomass invested in spines by seedlings of *Prosopis caldenia* and *Prosopis flexuosa* in the greenhouse study. Values are mean ± 1 SE ($N = 10$). The amount of biomass invested in spines was not affected ($P > 0.05$) either by species, water level, or fertilization level. There were no interactions ($P > 0.10$) among treatments.

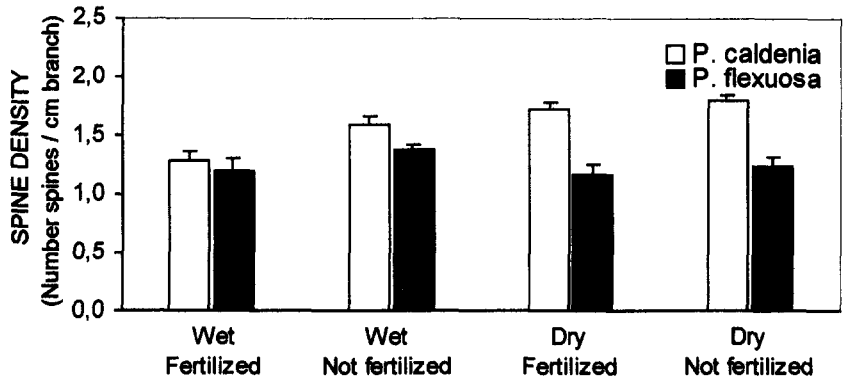

FIG. 5. Density of spines in seedlings of *Prosopis caldenia* and *Prosopis flexuosa* in the greenhouse study. Values are mean ± 1 SE ($N = 10$). Spine density was affected by species ($P < 0.01$), water level ($P < 0.05$), and fertilization level ($P < 0.01$). There was a species × water level interaction ($P < 0.01$).

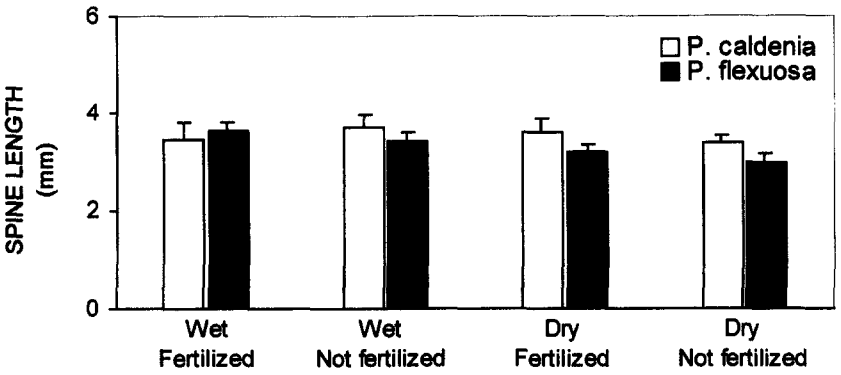

FIG. 6. Length of spines in seedlings of *Prosopis caldenia* and *Prosopis flexuosa* in the greenhouse study. Values are mean ± 1 SE ($N = 10$). Spine length was not affected ($P > 0.10$) either by species, water level, or fertilization level.

DISCUSSION

Based upon current antiherbivore defense theory, we predicted: (1) a higher investment in defenses in *P. flexuosa* than in *P. caldenia* and (2) that limitations in resources would result in an increase of the defenses in both species. The first prediction was based on the assumption that *P. flexuosa* has an inherent potential growth rate lower than *P. caldenia*. This assumption was supported

by results of the greenhouse experiment, where seedlings of *P. caldenia* grew faster than seedlings of *P. flexuosa* under conditions of high water availability and fertilization. Our results on phenol concentrations in leaves of adult plants growing in the field supported the first prediction: the concentration of phenols was higher in *P. flexuosa* than in *P. caldenia*. However, the concentration of phenols in leaves of juvenile plants growing under greenhouse conditions resulted in a pattern just the opposite of that observed for the adult plants growing in the field. A possible explanation for a higher level of defenses in seedlings of relatively fast growing genotypes has been provided by Bryant et al. (1991), who argued that genetic traits that favor a rapid increase in a seedling's photosynthetic capacity can shorten the time for expression of genes for juvenile-phase defense. A rapid increase in a seedling's leaf area and, therefore, photosynthetic capacity, favors the accumulation of carbohydrate reserves that can be used in the production of carbon-based defenses.

On the other hand, the similar proportion of biomass invested in spines in both adult plants and seedlings of *P. caldenia* and *P. flexuosa* did not support the first prediction. Moreover, spine density was higher in *P. caldenia* than in *P. flexuosa* in adults and seedlings. Other studies have also found that spinescence is prevalent in relatively fast-growing species from productive sites (reviewed by Myers and Bazely, 1991). The resource availability hypothesis may fail to explain interspecific differences in spinescence due to the fact that physical defenses are more expensive to produce than chemical ones, even when carbon is readily available (Skogsmyr and Fagerström, 1992). The same authors suggested consideration of the effects of herbivores and pathogens, in addition to resource availability, for improving the understanding of antiherbivore defense production. Concordantly, Milton (1991) argued that moisture may mediate mammalian herbivore selection of spinescence in moist, nutrient-rich habitats in arid areas where herbivores concentrate and plants are heavily broken, trampled, and browsed.

Our results did not offer support for the second prediction (limitations in resources result in increases in defenses). This prediction is based upon the assumption of a physiological trade-off between growth and defense production (secondary metabolism). However, neither in our field study nor in the greenhouse study was such a trade-off observed. In the field study, current season twig production was higher in the lowland site than in the upland site in 1993; however, the concentration of leaf phenols was higher in plants growing in the lowland site than in plants growing in the upland site, and all spinescent variables were similar in both sites. In the greenhouse study, despite the differences in biomass production among treatments, the concentration of leaf phenols, the amount of biomass invested in spines, and the length of spines were similar among treatments. In the seedlings of *P. caldenia*, spine density was inversely related to biomass production, but in the seedlings of *P. flexuosa* no such rela-

tionship was observed. Other studies where the levels of different resources were manipulated also have found no evidence to support the carbon–nutrient balance or the growth–differentiation balance hypotheses (Rousi et al., 1991; Dudt and Shure, 1994; McKee, 1995). We argue that the assumption of a direct trade-off between growth and defense may not be valid when alternative sinks (besides defenses) are competing for carbon. For example, in *P. caldenia* and *P. flexuosa* two alternative sinks are possibly N_2 fixation and accumulation of belowground reserves. The presence of nodules was observed in the root system of both species in all treatments under greenhouse conditions. Although there is no information on how much N_2 fixation may contribute to the N content of the studied species, N_2 fixation may contribute up to 61% to the N content of other species of the same genus (Shearer et al., 1983). It has been suggested that N_2 fixation is costly to the plant in terms of photosynthates that otherwise could be used in the production of antiherbivore defenses (Palo et al., 1993). On the other hand, although belowground reserves were not measured in the greenhouse study, the root–shoot ratio was higher at low water and nutrient availability, which may imply an increase in the storage of reserves under these conditions. Moreover, fire is apparently the main disturbance in the system where *P. caldenia* and *P. flexuosa* have evolved. As a result, these species have apparently been selected for traits such as large belowground reserves, which enable them to sprout again vigorously after fire (Bóo et al., 1997). Accumulation of carbohydrate reserves appears to start early in the ontogeny of the plants. For example, a high percentage of seedlings of *P. glandulosa* was able to resprout after being exposed to flame temperatures similar to those of hot grass fires (Wright et al., 1976). Thus, the allocation of carbohydrates to N_2 fixation and/or to belowground reserves may help explain the lack of trade-off between growth and production of defenses in *P. caldenia* and *P. flexuosa*. Moreover, if the species under study differ in N_2 fixation and/or in storage of reserves and/or in growth responses to stochastic events such as rainfall, these would be reasons (in addition to coming from habitats of different productivities) why they differ in defense allocation.

In general, our results did not support current hypotheses on genotypic and phenotypic variations in the production of antiherbivore defenses. From the preceding analyses, it appears that factors such as herbivore behavior (e.g., habitat selection, trampling, breaking) and alternative sinks for carbon other than growth and defense (e.g., N_2 fixation, carbohydrate reserves), in addition to resource availability in evolutionary and ecological time, need to be considered for a more complete and general understanding of the inter- and intraspecific variations in the production of antiherbivore defenses.

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OLFACTORY RESPONSES OF PARASITOID *Apoanagyrus lopezi* TO ODOR OF PLANTS, MEALYBUGS, AND PLANT–MEALYBUG COMPLEXES

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Abstract—*Apoanagyrus* (*Epidinocarsis*) *lopezi* De Santis is an endoparasitoid used in the biological control of the cassava mealybug *Phenacoccus manihoti* Matile-Ferrero in Africa. The response of naive and mated females of *A. lopezi* to odors from cassava plant (var. Zanaga), parasitized or unparasitized mealybugs, and plant–mealybug host complexes with or without feeding hosts was investigated in a Y-tube olfactometer. Dual-choice tests revealed that mealybug-infested plants and mealybug-damaged plants were the major sources of volatiles that attract female parasitoids to the microhabitat of its hosts. The emission of volatile chemicals appears not to be limited to the infested plant part but to occur systemically throughout the plant. On their own, unparasitized mealybugs were more attractive than uninfested plants or parasitized mealybugs alone. Parasitization of *P. manihoti* by *A. lopezi* decreased the response of parasitoids to mealybugs or mealybug–plant complexes. Plants infested with unparasitized hosts attracted more female parasitoids than plants infested with parasitized mealybugs. These results indicate that, in the long-range host-searching process, females of *A. lopezi* respond mainly to mealybug-induced synomones, and specific host-derived cues play a minor role.

Key Words—*Apoanagyrus lopezi*, *Phenacoccus manihoti*, cassava, biological control, resistance, olfactometer, herbivore-induced synomones, volatile chemicals.

INTRODUCTION

In the process leading to successful parasitization, the role of plant and host-derived volatile chemicals is of fundamental importance both in host-habitat

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location and host location by parasitoid insects (Vinson, 1976; Vinson and Williams, 1991; Weseloh, 1981). The efficacy of olfactory cues (plant- and host-derived volatiles) depends on their reliability in indicating herbivore presence, identity, accessibility, suitability, and detectability (Steinberg et al., 1993). Stimuli derived directly from the host are generally the most reliable source of information, but their effect is often limited to short distances, and their detectability at long ranges is low. Stimuli from host plants are usually released in large quantities but are less reliable as indicators of host presence (Vet et al., 1991). Plants that are infested by herbivores may emit so-called "herbivore-induced synomones" (Dicke and Sabelis, 1988; Vet and Dicke, 1992), which may combine the high detectability of plant volatiles with a high reliability in indicating herbivore presence through the specific interaction of the plant with the herbivore (Turlings et al., 1990a; Takabayashi et al., 1995). For plants, the specificity of the herbivore-induced synomones may be an important characteristic because it can ensure that the right natural enemy species is attracted (Takabayashi et al., 1995). The release of these chemical signals is not restricted to the infested plant parts, but occurs systemically throughout the plant (Dicke et al., 1990b; Takabayashi et al., 1991; Turlings and Tumlinson, 1992).

Apoanagyrus lopezi De Santis (Hymenoptera: Encyrtidae) is an endoparasitoid indigenous to South America that was introduced to sub-Saharan Africa in 1981 as a biological control agent against the cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae), which attacks cassava plants, *Manihot esculenta* Crantz (Euphorbiaceae). The cassava mealybug is a parthenogenetic species with three instars. It is a phloem-feeding insect that secretes honeydew while feeding (Calatayud et al., 1994). *A. lopezi* is monophagous on *P. manihoti*, which is restricted to cassava. These cassava–mealybug and mealybug–parasitoid systems have been studied extensively, both in the laboratory and under field conditions (Fabres, 1981; Löhr et al., 1988; Neuenschwander et al., 1990; Nénon, 1992); data are still lacking on tritrophic aspects of cassava mealybugs, and there is no information on interactions between cassava and *A. lopezi*. Souissi and Le Rü (1997a,b) reported that the biological parameters of the parasitoid are influenced by cassava cultivars characterized by different levels of antibiosis resistance. Reports on *A. lopezi* searching behavior are also previously mentioned but are not in full agreement. In fact, Nadel and van Alphen (1987) demonstrated that females of *A. lopezi* are attracted by mealybug-infested plants but not by the odor of hosts alone or by uninfested plants, whereas van Baaren (1994), reported that female parasitoids are attracted by odors emanating from unparasitized hosts or from uninfested plants. Moreover, the exact origin of the stimuli that affect the long-range host-searching behavior of this parasitoid is still unknown.

The aim of the present study was to investigate in a Y-tube olfactometer the response of individual females of *A. lopezi* to volatile chemicals emitted

from components of the first and second trophic level of the cassava–mealybug complex. We studied the relative importance in the host-searching process of olfactory stimuli from the mealybug, the healthy plant, and the plant–host complex with or without feeding mealybugs. Dual-choice tests were also made to compare the attractiveness of the host and/or plant using parasitized or unparasitized mealybugs to determine whether parasitization of *P. manihoti* by *A. lopezi* affects the response of female parasitoids to volatiles released by the host or by the infested plant, and whether these olfactory stimuli were involved in the detection of parasitized hosts and parasitized host-infested plants. We further tested whether the production of volatile chemicals is restricted to the infested part of a plant or occurs systemically throughout the plant.

METHODS AND MATERIALS

Plants. The host plant Zanaga (a cassava variety) came from the Congo. Plants were obtained from cuttings 20 cm high, planted in pots with two thirds of their length in soil, and placed in a controlled room at 25 ± 1°C, 12L:12D, and 70 ± 3% relative humidity. Light intensity was 700–800 lux (artificial light source). They were watered twice weekly. Plants 20–30 cm in height were used for the experiments.

Insects. The mealybugs and parasitoid strains came from the Congo. The mealybug, *P. manihoti*, was reared on the Zanaga cassava variety. Mealybugs at the L3 stage, the preferred stage for female parasitoids (Löhr et al., 1988; Iziquel, 1990), were used in experiments.

A. lopezi was reared on mealybugs fed different cassava varieties from Benin and Burkina Faso. Adults were supplied with honey droplets as food. For experiments, mummies were harvested from the parasitoid culture and kept individually in clear gelatin capsules. Upon emergence, female parasitoids were placed singly with two or three males for 24 hr in clear polystyrene tubes (15 × 1.5 cm) where they had honey available. All experiments were carried out with naive (no oviposition and no contact with cassava plants), mated females that were 1–3 days old.

Mealybugs and parasitoids were maintained in separate controlled insect rearing rooms at 25 ± 1°C, 12L:12D and 70 ± 3% relative humidity.

Olfactometer Set-Up. The response of females of *A. lopezi* to volatile chemicals emitted by different odor sources was investigated in a Y-tube olfactometer. The olfactometer consisted of a Y-shaped glass tube 2.5 cm in diameter. The base and the two arms of the Y tube were 12 and 13 cm in length, respectively. Each arm was connected to a flowmeter and an odor source container, which consisted of a glass cylinder, 25 cm in diameter and 40 cm in height, large enough to hold a whole potted cassava plant. Parafilm was used to cover the

soil of the pot, thus isolating it from plant foliage through which the airstream was blown. With air pressure, an airflow was generated through an humidifier bottle. The humidified airflow was divided in two, and each subflow was led through an odor container. Subsequently, the two odor flows were led through the two arms of the Y-tube olfactometer. The airflow through each olfactometer arm was 10 liters/hr and was checked with a flowmeter. Female parasitoids were able to walk upwind towards the arms of the tube and sometimes performed short flight attempts. For more details on the olfactometer, see Steinberg et al. (1992). The experiment was done at 25 ± 1°C and 70 ± 5% relative humidity in a box painted white with an artificial light source consisting of a single 35-W fluorescent tube placed above the arms of the Y tube.

Bioassay Procedure. Naive and mated female parasitoids were introduced individually into the base tube of the Y-olfactometer and given 4 min to walk towards the end of one of the arms of the olfactometer. When the female crossed the choice line 4 cm after the division of the base tube and remained there for at least 20 sec, it was recorded as a choice for the odor source in that arm. The connections of the odor source container to the olfactometer arms were exchanged after testing five parasitoids to remove any asymmetrical bias of the set-up. The olfactometer tube was washed with alcohol after testing 10 females. Tests were carried out 2–5 hr after the beginning of the photophase, which corresponds to the most active period of *A. lopezi* (van Baaren, 1994). A total of 40 females were tested in each choice situation. The small number of females giving no response was neglected. The choice of the females was analyzed with a chi-square test ($P = 0.05$).

Odors Sources. Ten odor sources were tested: (1) clean air, which consisted of an empty odor source container. (2) An uninfested plant—plants were approximately 8–12 weeks old (8–10 leaves, 20–30 cm high) and were used as a standard clean cassava odor source. (3) The complete mealybug-infested plant—plants were infested with 20 unparasitized third instar mealybugs placed in groups of 5 on four different leaves. Leaves were chosen randomly, and the test was run three days after infestation to enhance the damage done by the mealybugs. (4) Complete mealybug-infested plant from which mealybugs and honeydew had been removed with a paintbrush and leaves had been washed with distilled water prior to testing. (5) Twenty unparasitized mealybugs removed from a plant approximately 20 min prior to testing. (6) Twenty unparasitized mealybugs removed from a plant 24 hr prior to testing. (7) Twenty parasitized mealybugs removed from a plant approximately 20 min before the beginning of the test. Mealybugs on a cassava plant were exposed to 10 female parasitoids during a 24-hr period. In these conditions, more than 95% of hosts were parasitized, as was later demonstrated by dissecting mealybugs (five groups of 20 mealybugs prepared in the same manner) (van Baaren, 1994). For sources 5–7, mealybugs removed from a cassava plant were kept in glass vials (5 × 1.2 cm).

(8) A cassava plant infested with 20 parasitized mealybugs—the plant was infested with 20 mealybugs that had been previously exposed to 10 mated female parasitoids for 24 hr. The test was run three days after infestation. (9) Uninfested clean leaves removed from an infested plant 72 hr after infestation—these were produced by enclosing two to three leaves per plant in fine mesh bags and infesting the remainder of the plant using the L3 mealybugs. (10) Clean leaves from a clean plant. For sources 9 and 10, leaves from each plant were picked with their petiole and placed in a glass vial (10 cm high, 2 cm in diameter) filled with 20 ml distilled water. Parafilm was used to seal the vials. Leaves in vials were placed in the glass cylinder of the olfactometer where the airstream was blown.

RESULTS

Effect of Infestation on Response of Parasitoid A. lopezi. Naive female parasitoids showed no response towards an uninfested plant compared to clean air. When an infested plant was placed in the olfactometer and compared to an uninfested plant or to mealybugs removed from the plant 20 min prior to testing, the majority of parasitoids showed a preference for the infested plants (Figure 1).

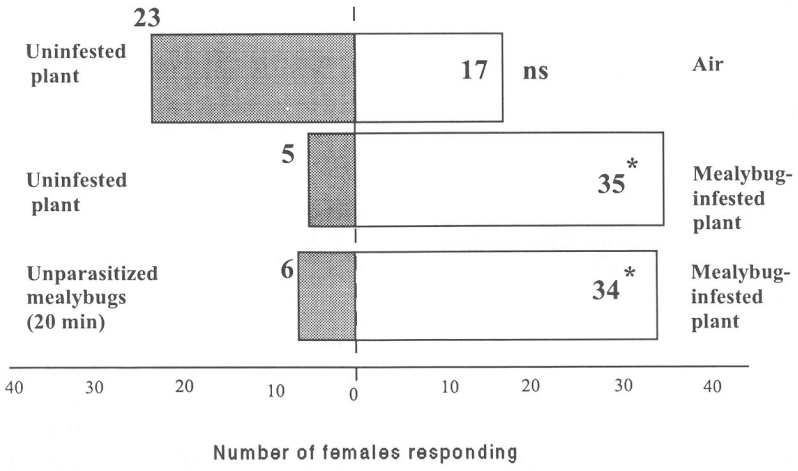

FIG. 1. Olfactory responses of *A. lopezi* females to an uninfested plant tested against air and to a mealybug-infested plant tested against an uninfested plant and mealybugs (20 min) alone in a Y-tube olfactometer. Asterisks indicate a significant difference between a choice test: $*P = 0.05$; ns = not significantly different at $P = 0.05$ (chi-square test). Numbers at right- and left-hand sides of figure indicate the number of females that made a choice for each one of the two odor sources offered.

A mealybug-damaged plant attracted a significantly higher number of parasitoids than did an uninfested plant or one which had mealybugs removed 20 min before the test. When a mealybug-damaged plant was tested against an infested plant, *A. lopezi* did not show a significant preference for either of the two odor sources offered (Figure 2). Furthermore, *A. lopezi* females found clean leaves from an infested plant more attractive than those from an uninfested plant (Figure 2).

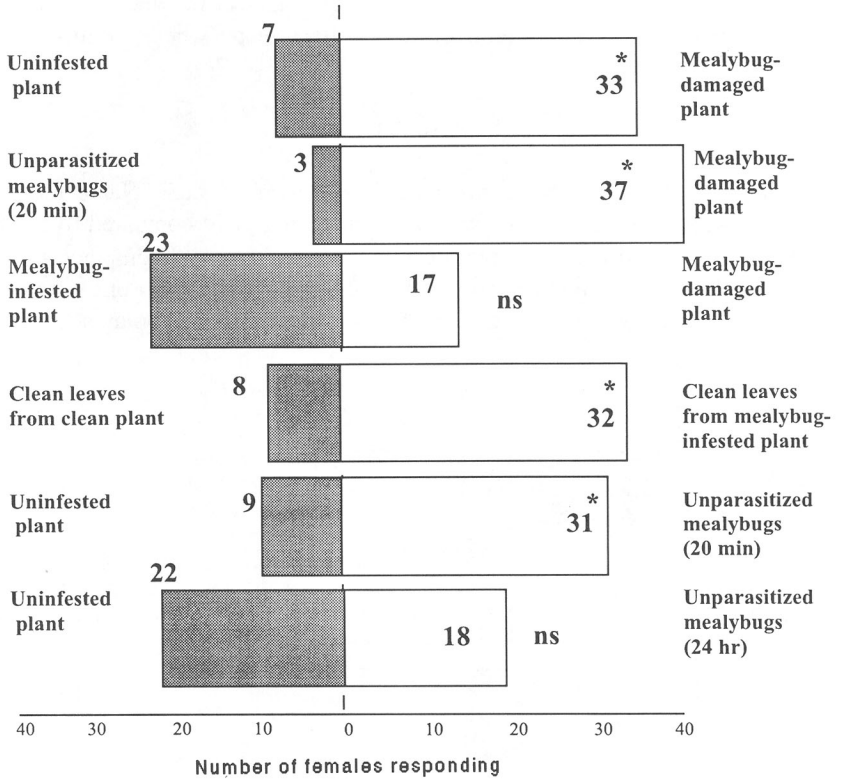

FIG. 2. Olfactory responses of *A. lopezi* females to a mealybug-damaged plant tested against three odor sources—an uninfested plant, unparasitized mealybugs (20 min) and a mealybug-infested plant—and to clean leaves from a mealybug-infested plant tested against clean leaves from an uninfested plant and to an uninfested plant tested against unparasitized mealybugs removed from plant 20 min and 24 hr prior to testing. Asterisks indicate a significant difference between a choice test: $*P = 0.05$; ns = not significantly different at $P = 0.05$ (chi-square test). Numbers at right- and left-hand sides of figure indicate the number of females that made a choice for each one of the two odor sources offered.

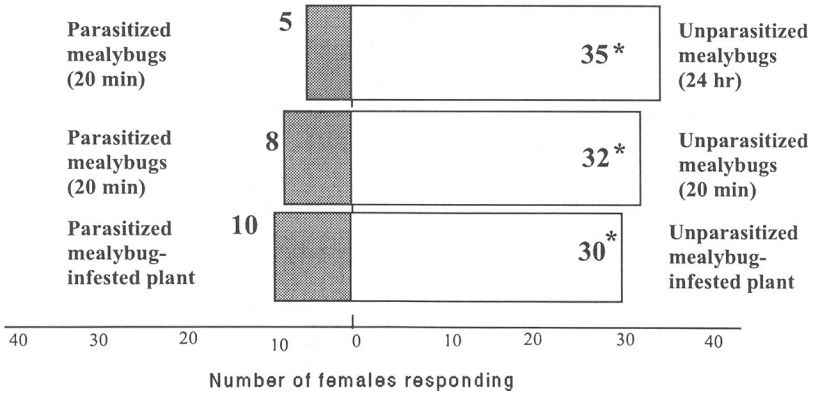

FIG. 3. Olfactory responses of *A. lopezi* females to unparasitized mealybugs (24 hr and 20 min) and to an unparasitized mealybug-infested plant tested against parasitized mealybugs alone and a parasitized mealybug-infested plant, respectively. Asterisks indicate a significant difference between a choice test: $*P = 0.05$; ns = not significantly different at $P = 0.05$ (chi-square test). Numbers at right- and left-hand sides of figure indicate the number of females that made a choice for each one of the two odor sources offered.

Naive female parasitoids preferred the odor emitted by unparasitized mealybugs removed 20 min before the test to that of an uninfested plant. In contrast, *A. lopezi* females did not show a response towards mealybugs removed from the plant 24 hr prior to testing when compared to an uninfested plant (Figure 2).

Effect of Parasitization on Attractiveness of Mealybugs and Mealybug-Infested Plants. Female parasitoids showed a clear preference for unparasitized mealybugs removed from a plant 20 min or 24 hr before the beginning of the test when compared to parasitized ones. In addition, females gave a significantly greater response to a plant infested with unparasitized mealybugs than to a plant infested with parasitized mealybugs (Figure 3).

DISCUSSION

Females of *A. lopezi* did not respond to uninfested cassava plants in the olfactometer when compared with clean air, a mealybug-infested plant, or a mealybug-damaged plant. Parasitoids did not distinguish between a *P. manihoti*-infested plant with feeding mealybugs or a *P. manihoti*-infested plant from which the mealybugs and host by-products had been removed, whereas they highly

preferred these odor sources to mealybug odor alone. These results indicate that mealybug-infested or mealybug-damaged plants are the primary source of the volatile chemicals that attracted *A. lopezi* and guided it to the host habitat. This result is consistent with those reported in earlier studies with other parasitoid species. Each of the six species of aphid parasitoids tested by Wickremasinghe and van Emden (1992) showed an odor preference for the aphid–plant complex rather than for the uninfested plant alone. Likewise, Steinberg et al. (1993) showed that the plant–host complex was the most attractive odor source for parasitoids in their experiment with *Cotesia glomerata* (L.).

Damage by herbivores can increase enormously the emission of plant volatiles (Dicke et al., 1990a). In our study, the increased response of *A. lopezi* to a mealybug-infested cassava plant could be due to increased production of volatiles or to a changed volatile blend that was more attractive. In addition, our results indicate that the release of these chemical volatiles is not restricted to the mealybug-infested parts but occurs systemically throughout the plant. In our study, females of *A. lopezi* were attracted by undamaged leaves of the mealybug-infested plant. Similar results have been found in other tritrophic systems (Dicke et al., 1990b; Takabayashi et al., 1991; Turlings and Tumlinson, 1992). This suggests that an attractive element is produced by mealybugs or the plant itself at the site of infestation in leaves from infested plants, and that its effects appear in clean leaves isolated from other parts of the infested plant. *P. manihoti* is a phloemophagous insect on cassava, and the ingestion with injection of saliva occurs strictly in phloem sap (Calatayud et al., 1994). Thus, it is possible that buccal secretions or salivary enzymes yield product at the site of infestation which are then translocated in the phloem sap to other leaves, as previously suggested (Turlings et al., 1990a; Dicke et al., 1993). Cassava undergoes physiological changes in response to mealybug infestation, as shown by a significant increase in the levels of three flavonoid glyucosides (Calatayud et al., 1994) and by a change in the cyanide content of leaves, stems, and tubers (Ayanru and Sharma, 1985). Further investigations are needed to determine whether the different levels of cyanogenic and flavonoid glycosides observed after infestation influence the production of synomones in cassava. *A. lopezi* showed no preference for mealybugs removed from the plant 24 hr prior to testing over an uninfested plant. This may support a plant origin of compounds that are emitted by mealybugs removed from a plant 20 min before the test.

The attractiveness of unparasitized mealybugs appeared significantly lower than that of mealybug-infested plants with or without feeding mealybugs. This result indicates that females of *A. lopezi* are not attracted at long range by mealybug kairomones but are attracted directly to mealybug-infested plants. Cues from host by-products, such as wax and honeydew, seem to operate at close range once female parasitoids have landed on plants. Langenbach and van Alphen (1986) have shown that *A. lopezi* reacted to a kairomone present in the

wax of mealybugs and spent more time preening on wax-contaminated leaves than on clean leaves.

In our experiment, unparasitized mealybugs removed from plants 20 min prior to testing were more attractive than uninfested plants. This positive response might be due to volatiles emitted from the cassava plant and not from mealybugs themselves. For the test, mealybugs were removed from the plant approximately 20 min before the beginning of the test, and, perhaps, this time interval was not sufficient to dissipate volatile substances contaminating mealybugs that were attractive to *A. lopezi* in the olfactometer. Read et al. (1970) reported a similar result with females of the braconid *Diaeretiella rapae* (M'Intosh), which were attracted to the cabbage aphid *Brevicoryne brassicae* (L.) freshly removed from collards (15 min before the test) but not to aphids removed from the host plants 24 hr before the test.

Nadel and van Alphen (1987) reported that females of *A. lopezi* were attracted in a four-armed olfactometer by the odor of mealybug-infested cassava leaves (unspecified variety), but not by the odor of cassava mealybugs alone or by uninfested leaves. In contrast, van Baaren (1994) reported that in a tubular olfactometer *A. lopezi* females were attracted by the odor of host-plant (M'Pembé cassava variety) alone or by unparasitized mealybugs. These results differ in part from each other and from ours. Discrepancies between results could be due to a different variety of cassava or to a different experimental procedure. The composition of volatile blends emitted by plants is mostly plant-determined and may depend on the species or variety and on the quality, which is determined by age and growing conditions (Takabayashi et al., 1991, 1994). Thus, it is likely that the whole Zanaga casava variety used in our tests produced larger quantities of volatiles than did isolated plant parts, such as leaves (Nadel and van Alphen, 1987) or apex (van Baaren, 1994). It may be assumed that overall a greater response from female parasitoids was obtained in our experimental set-up. Nevertheless, female parasitoids used in our study had no previous experience with either mealybugs or cassava plants. In Nadel and van Alphen's experiments, females of *A. lopezi* were collected daily from the cages in which they emerged and therefore had experienced the odors of mealybugs and cassava plants. It is possible that one leaf removed from an uninfested plant or a group of mealybugs brushed from leaves were not sufficient to elicit a positive response in parasitoids, which prior to the experiment had been subjected to much greater intensities and quantities of volatiles within the confines of the rearing cage. In van Baaren's experiments, females of *A. lopezi* also gained experience by being exposed to hosts 24 hr before the test. Previous experience with either host or plant has been reported to enhance the response of parasitoids to that tested component of the plant–host complex (Sheehan and Shelton, 1989; Turlings et al., 1990b). Therefore, it is possible that results reported previously were amplified by the previous experience of *A. lopezi*.

In our study, unparasitized mealybug-infested plants and unparasitized mealybugs alone were found more attractive to *A. lopezi* than plants infested with parasitized mealybugs and parasitized mealybugs alone. Parasitized insects inflict less damage on a plant than unparasitized ones (Turlings et al., 1991; Takabayashi et al., 1995). Parasitization of mealybugs by *A. lopezi* probably reduces the feeding of mealybugs, and so the damage to the plant is less important. As a result, the amount of synomones produced by a mealybug-infested plant might also be reduced. It seems, therefore, that the chemicals produced by the plant in response to the damage inflicted by specific herbivores would be particularly attractive to parasitoids in the early stage of infestation and thus beneficial to plants (Takabayashi et al., 1995). Van Baaren (1994) reported that the odor of parasitized mealybugs on the host-plant induced an increase in the search activity of *A. lopezi*, but it was also found to be repellent. This effect would explain the preference of *A. lopezi* for odors emitted by unparasitized hosts. It might be advantageous to a parasitoid if it favors its dispersion towards unexploited parts of the habitat or if the parasitoid spends more time and effort searching in a patch where only a few hosts are already parasitized.

Our data suggest that the source of attractants for females of *A. lopezi* originates from the interaction between the plant and the feeding of mealybugs, i.e., herbivore-induced synomones (Vet and Dicke, 1992). These chemicals can be detected at longer distances than those produced by undamaged plants or kairomones left by unparasitized hosts. Their role might be to guide parasitoids into areas that harbor hosts. Olfactory stimuli from hosts seem to play only a minor role in the long-range host-searching behavior of this parasitoid. They appear to have an arrestant effect and stimulate foraging females to search for hosts after landing on infested plants (Langenbach and van Alphen, 1986).

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LABORATORY EVALUATION OF PREDATOR ODORS FOR ELICITING AN AVOIDANCE RESPONSE IN ROOF RATS (*Rattus rattus*)

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Abstract—We evaluated eight synthetic predator odors and mongoose (*Herpestes auropunctatus*) feces for eliciting avoidance responses and/or reduced feeding by wild captured Hawaiian roof rats (*Rattus rattus*). In a bioassay arena, we recorded: (1) time until each rat entered the arena, (2) time elapsed until first eating bout, (3) time spent in each half of the arena, (4) number of eating bouts, and (5) consumption. Rats displayed a response to the predator odors in terms of increased elapsed time before initial arena entry and initial eating bout, a lower number of eating bouts, and less food consumption than in the respective control groups. The odor that produced the greatest differences in response relative to the control group was 3,3-dimethyl-1,2-dithiolane [from red fox (*Vulpes vulpes*) feces and mustelid anal scent gland]. Mongoose fecal odor produced different responses in four of the five variables measured while (*E,Z*)-2,4,5-trimethyl-Δ^3-thiazoline (red fox feces) and 4-mercapto-4-methylpentan-2-one (red fox urine and feces) odors were different from the control group in three of the five variables measured. These laboratory responses suggest that wild Hawaiian roof rats avoid predator odors.

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Key Words—Laboratory bioassay, *Rattus rattus*, roof rats, avoidance behavior, mustelids, anal-gland compounds, red fox urine, feces, *Vulpes vulpes*, mongoose, *Herpestes auropunctatus*.

INTRODUCTION

The macadamia nut (*Macadamia integrifolia*) is a desirable food item internationally and is a crop of high commercial value. Crop damage by rodents has been a problem on the Hawaiian Islands for many years, and annual losses have been estimated at $3–4 million (Tobin et al., 1993). The roof rat (*Rattus rattus*) has been identified as the primary pest species in macadamia nut orchards, with the Polynesian rat (*R. exulans*) also present, but not considered as a serious damage agent (Tobin, 1992). Various management techniques have been applied, but none has proven effective.

Current rodent control techniques in Hawaii involve poisoning (rodenticides) and/or the use of kill-traps. Studies on the effectiveness of common rodenticides have shown many poisons to be too selective for certain rat species (Tobin, 1992). This selectiveness is related to increased zinc phosphide use and is believed to explain the increase in Norway rat (*Rattus norvegicus*) populations relative to the roof and Polynesian rat (*Rattus exulans*) in sugarcane fields (Tobin et al., 1990). Another basic problem with zinc phosphide is bait shyness resulting from sublethal consumption of the bait. As macadamia growers need to protect their crop over an extended period of time, this learned bait avoidance becomes a problem. Timing of application in the orchard is important, and restrictions regarding the use of zinc phosphide prior to harvest are currently required, primarily to reduce crop residues and primary/secondary accidental poisoning of humans. The potential effects on other nontarget animals (e.g., feral pigs, avian predators) and possible transmission through the food web are also of concern with anticoagulant rodenticides (Engeman and Pank, 1984).

The use of synthetic predator semiochemicals in wildlife management is a growing area of research. Although pheromones have been used extensively in agricultural pest management, the focus has been primarily on invertebrate communities. The application of predator odors as a management tool at the small mammal level has been recently investigated. Sullivan et al. (1988a–c) have shown suppressed damage and/or an avoidance response in synthetic predator odor experiments with various small mammal populations in both agricultural and forested ecosystems. Significant results have been observed in the following North American species: an avoidance response in snowshoe hare (*Lepus americanus*) in coniferous seedling plantations, suppressed feeding by montane and meadow voles (*Microtus montanus* and *M. pennsylvanicus*) in apple tree orchards, and an avoidance response by northern pocket gophers (*Thomomys talpoides*) in apple tree orchards.

The encouraging results from the previously described odor response studies, combined with an extensive rodent problem on the Hawaiian Islands, provided an ideal situation in which to explore potential management applications. A recent field study in Hawaii also indicated that rats avoid traps scented with mongoose feces (*Herpestes auropunctatus*) (Tobin et al., 1995). We examined the possibility of using synthetic predator odors to prevent feeding damage by rats in a macadamia nut orchard. This biological control method attempts to prevent rodent damage through a behavioral response to a predator odor. Although most of the odors tested were not those of established predators on the Hawaiian Islands, we predicted that a fear/avoidance response should be innate. Volatiles from red fox (*Vulpes vulpes*) fecal droppings elicited a fear response in Wistar lab rats that had not been in contact with predators for generations (Vernet-Maury et al., 1984). Boag and Mlotkiewicz (1994) found decreased rabbit numbers in areas treated with a complex multicomponent synthetic repellent derived from lion feces. Self-anointing behavior with weasel (*Mustela sibirica*) anal scent gland secretion, considered a defensive response, was displayed by juvenile rice-field rats (*Rattus rattoides*) that had been lab reared (Xu et al. 1995).

We based our predictions of roof rat response to predator odors on previous studies involving other small mammal species. Vernet-Maury et al. (1992) assessed fear response observations of wild-caught Norway rats (*Rattus norvegicus*) in an open-field arena, measuring time of emergence, exploratory movements, grooming, urination, and defecation. Other studies found reduced food consumption by small mammals in response to predator odors (Calder and Gorman, 1991; Epple et al., 1993; Heale and Vanderwolf, 1994). Based on the success of these various studies and the apparent innate responses by small herbivores to carnivore odors, we predict that roof rat behavior can be manipulated with synthetic odors in the laboratory.

This study was designed to test the hypotheses that presence of a predator odor will: (1) increase the time elapsed before an individual first enters the arena and before its first eating bout for the treatment groups relative to the control group: (2) reduce the amount of time spent on the treated side relative to the control side and reduce the total time spent on both sides of the arena; (3) reduce the number of eating bouts on the treated side relative to the control side; and (4) reduce the amount of food consumed on the treated side relative to the control side.

METHODS AND MATERIALS

An open-box arena design allowed a large viewing area to observe an individual rat's response. A video camera (JVC BY-1000) situated above the

arena recorded the information and eliminated observer bias. As a plantation management interest was to reduce feeding damage, consumption became one of the response variables measured. Other quantitative variables measured were time to first entry, time to first eating bout, time spent in arena, and number of eating bouts. Separating the open box arena into two identical halves allowed a predator odor to be tested against a control.

Capture and Maintenance of Animals. Roof rats were live-trapped in the Waikaea Forest Reserve on the island of Hawaii and maintained in animal rooms at the United States Department of Agriculture (USDA)/Animal and Plant Health Inspection Service (APHIS)/Animal Damage Control (ADC)/Denver Wildlife Research Center (DWRC) field station in Hilo. Only adult animals (sexually mature and weight ≥ 90 g) were maintained for testing. Over 120 roof rats were initially captured, from which 100 animals (50 of each sex) were randomly selected to meet the study design requirements of 100 animals. Test animals were housed in individually numbered cages and were provided with water and laboratory chow *ad libitum* prior to testing. The animals were maintained on a 12L:12D schedule with the room temperature ranging from 20° to 22°C.

Mongooses, maintained to collect fresh fecal samples, were captured in gulch regions near the city of Hilo and were also housed at the DWRC Hilo Field Station. Two adult mongooses, one of each sex, were maintained in an outdoor wire pen and provided with shelter, bedding, water *ad libitum*, and fed one rat per day.

Assignment to Treatment Groups. The 100 test rats were assigned to 10 treatment groups using a randomization program that ranked each sex group by weight before randomly assigning treatments. Following the treatment assignment, each test group consisted of five males and five females. As the odor trials were divided into two phases, each treatment group was further divided into two subgroups (two males/three females or three males/two females). The test animals were then housed according to treatment group and subgroup (A or B).

Arena Design. The size of the arena was maximized according to the largest field of view allowable with the video camera lens. The arena measured 150 cm long × 60 cm wide × 120 cm high (Figure 1). A middle wall divided the box into two equal areas with a small opening (15 × 15 cm) located in the bottom corner to allow access between both sides of the arena. The side of the arena with the opening in the middle wall also had the only opening to outside the arena, a 10-cm × 10-cm opening with an outer sliding door. Brackets were secured on either side of the door to permit the animal transfer cage to be directly attached to the arena. Once the animal transfer cage was attached, the sliding door could be opened to allow the test animal access to the arena.

As some of the odors tested were very volatile, materials to construct the arena were chosen based on their properties of low absorption and ease of

cleaning. The arena walls, door, and animal transfer cage were constructed of stainless steel. The arena had no bottom and was set on a sheet of white Formica. Following any animal trial, the arena was cleaned with a combination of bleach/detergent/water, then rinsed with water and sprayed with ethanol to evaporate any residual odors. The arena and camera were located in a separate room from the animal housing room, where temperature, lighting, and air circulation could be controlled separately.

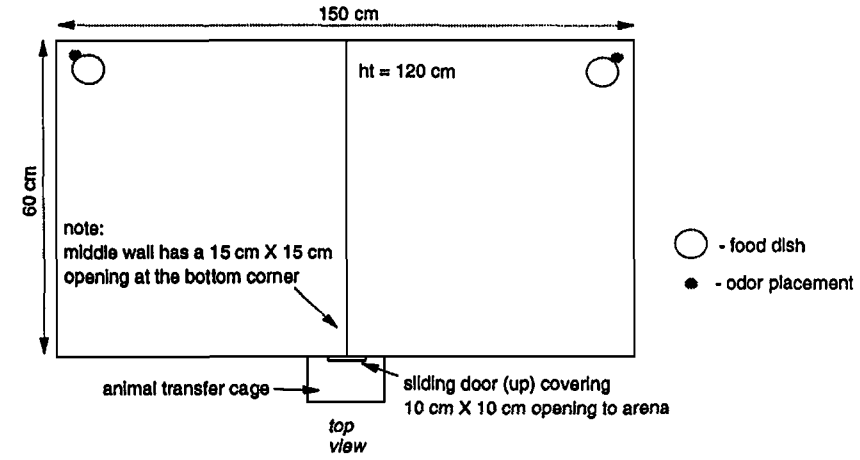
The video camera was situated on a tripod directly above the middle of the arena. A television located outside the testing room was used for live viewing. During video recording, a low-intensity red-filtered light simulated nocturnal conditions under which the animals are normally active.

Test Procedure. Following pilot trials with nontest animals, it was decided to test and record individuals over a 60-min period. Only one predator odor was tested per day to reduce possible residual odor effects. This design allowed one odor and five individuals to be tested per day during the 12-hr dark phase (1800–0600 hr) when the animals are normally more active. The procedure was divided into two phases encompassing 10 days each. Each phase was identical in procedure and order of odor compounds tested. This schedule was chosen to try to prevent any effect that may occur with time, as individuals tested in the final trials will have been housed for three weeks longer than those tested initially. The phases were also scheduled as close together as possible to further reduce any time effects.

Each test animal was preexposed to the arena on the two days prior to the test day. This procedure decreased any novel effect the arena environment may have had on the individual. The preexposure trials were 15 min in duration and were also recorded on video tape. The only differences in preexposure trials from odor trials were a shorter duration, an absence of odor compounds, no prior food deprivation, and they were performed during the latter quarter of the 12-hr light period.

Prior to the day of odor testing, animals were food-deprived for 24 hr to increase their motivation to feed. This is a common procedure in laboratory trials investigating rodent behavioral responses (R. J. Blanchard, personal communication).

For the odor testing, 10 μl of a given compound were pipetted into a urethane vial, with the odor released through a small hole in the cap of the vial. The only exception to this procedure was for the mongoose feces treatment, in which $\sim$1 g was placed directly into the vial with two drops of water ($\sim$0.02 ml). The odor vial was attached to the outside of a bowl that contained a measured quantity of coconut bait. The bowls were then placed in the corners of the arena, against the wall opposite from the animal transfer cage (Figure 1). Each trial had one vial containing the test chemical and the other containing water. For each treatment group, the placement of the test odor was randomly



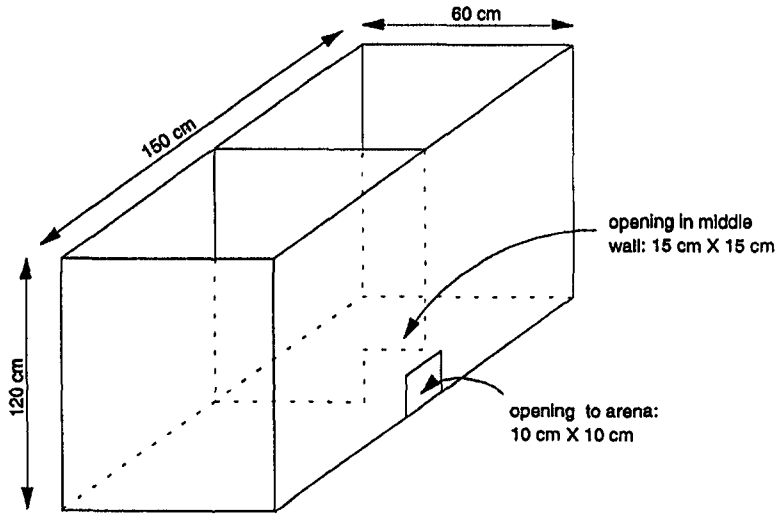


FIG. 1. Laboratory testing arena design for evaluation of predator odors to elicit an avoidance response in the roof rat (not to scale).

located for the first subgroup and placed on the opposite side for the second subgroup. This assignment reduced any bias for either side.

On the test day, the individual was moved from the animal room to the test room with the animal transfer cage. The individual was left undisturbed for 4 min to allow the subject to rest following the transfer procedure. The duration of rest time was determined based on discussions with Dr. R. J. Blanchard (personal communication) and time constraints. Using the television, the observer would record information while the animal was being video-taped, while further data were recorded during subsequent review of the tapes. Each test animal was recorded for 60 min after opening the sliding door. After each trial, the animal was returned to the animal housing room, and the arena dismantled for cleaning. The coconut bait was checked for signs of feeding and reweighed to measure consumption during the trial.

Predator Odors. Chemical compounds to be tested as repellents were originally derived from predator species, commonly from the anal scent gland, urine, or feces. The compounds have generally been identified either from extracts of these secretions or from the volatiles that emanate from them. The components believed to have semiochemical activity were then prepared synthetically. All of the odor constituents (semiochemicals) tested were synthetic liquids except for mongoose feces, which were collected from freshly voided material. The semiochemical compounds were synthesized at $>90\%$ purity by Industrial Research Limited, New Zealand and Phero Tech Inc., Delta, British Columbia, Canada. A list of the odors, an abbreviation, and their original source are given in Table 1.

Statistical Analysis. The results were analyzed as a fixed-effect randomized-block design with sex being a blocked factor. Blocking sex was performed because previous laboratory studies with the roof rat found differences between male and female consumption (Sugihara et al., 1995). For the variables that took into account the "side" factor, a three-factor repeated measures analysis of variance (ANOVA) was used with sex and treatment as the two nonrepeated factors. Variables that did not use side as a factor were analyzed with a two-factor ANOVA (sex and treatment). See Table 2 for details regarding the specific analysis of the variables measured.

The univariate repeated measures analysis has similar assumptions to the regular ANOVA. However, this analysis also assumes circularity among the levels of within-subject factor (von Ende, 1993). The within-subject factor in this case was "side," and as there were only two levels of this factor, we assumed that the difference between these two factors equaled the same value for each treatment level. This was based on the graphical representation of the descriptive statistics "by side." As the sequence of treatment levels was random and we assumed no carryover effects on the variables measured from one treatment to the next, the circularity assumption was probably met (von Ende, 1993).

TABLE 1. CHEMICAL NAME, ABBREVIATION, AND SOURCE OF PREDATOR ODORS TESTED IN LABORATORY EVALUATION

Chemical name	Abbreviation	Source
2,2-Dimethylthietane	DMT	mustelid (*Mustela* spp.) anal scent gland
3,3-Dimethyl-1,2-dithiolane	DMDIT	mustelid (*Mustela* spp.) anal scent gland red fox feces
Isopentenyl methyl sulfide	IPMS	red fox urine
Herpestes auropunctatus feces	MONG	mongoose feces
4-Mercapto-4-methylpentan-2-one	MMP	red fox urine and feces
(±)-3-Propyl-1,2-dithiolane	PDT	stoat (*Mustela erminea*) anal scent gland
(±)-2-propylthietane	PT	stoat (*Mustela erminea*) anal scent gland
2-*sec*-butyl-Δ^2-thiazoline	SBT	mouse aggressor hormone
(*E*,*Z*)-2,4,5-Trimethyl-Δ^3-thiazoline	TMT	red fox feces

The "time spent" variable was analyzed as two-factor nonrepeated ANOVA because the temporal nature of the data would violate an assumption in the repeated measures analysis. As the time a subject spends on one side is inversely related to the time it spends on the opposite side, the side data becomes less independent (R. Engeman, personal communication). The level of significance (α) was set at the 0.05 level for all ANOVAs. All statistical analysis was performed with the statistical program SAS (SAS Institute, 1988).

TABLE 2. VARIABLES MEASURED AND ANALYSIS TECHNIQUE USED TO EVALUATE LABORATORY TESTING OF PREDATOR ODORS

Variable	Analysis method
Time to emerge	two-factor ANOVA
Time to first eating bout	two-factor ANOVA
Total time spent in arena	two-factor ANOVA
Number of eating bouts	three-factor repeated measures ANOVA
Consumption	three-factor repeated measures ANOVA

RESULTS

Arena Bioassays. Although significant differences were not detected at $\alpha = 0.05$ ($P = 0.13$) for time elapsed to arena entry, the data were graphed with sexes grouped to assess trends (Figure 2). The mean (± SE) for entry times of rats exposed to DMDIT (explanations of abbreviations here and following can be found in Table 1) (97 ± 23 sec) and IPMS (93 ± 27 sec) were greater than for rats in the control group (39 ± 16 sec).

For time elapsed to first eating bout, no significant difference was detected between treatment groups ($P = 0.19$). Again, the data were graphed to assess potential trends (Figure 3). The mean (± SE) elapsed times to first eating bout for rats exposed to DMDIT (20.6 ± 7.2 min), MMP (19.3 ± 7.4 min), MONG (15.7 ± 7.1 min), PT (15.1 ± 6.4 min), SBT (17.3 ± 6.2 min), and TMT (21.1 ± 6.8 min) were greater than that for rats in the control group (6.2 ± 2.5 min). These six treatment groups were also the only treatments with animals that did not eat throughout the odor trial.

There were no significant differences ($P = 0.16$) found with respect to the time spent variable by side or by treatment. Because there were no side or sex differences for the total time spent, the data were sex and side grouped (Figure 4). This described the amount of time spent in the arena as opposed to within the covered transfer cage. There were observable trends in the amount of time spent in the arena between treatment groups and the control. The mean (± SE) times spent in the arena for rats exposed to DMDIT (20.0 ± 3.4 min), DMT

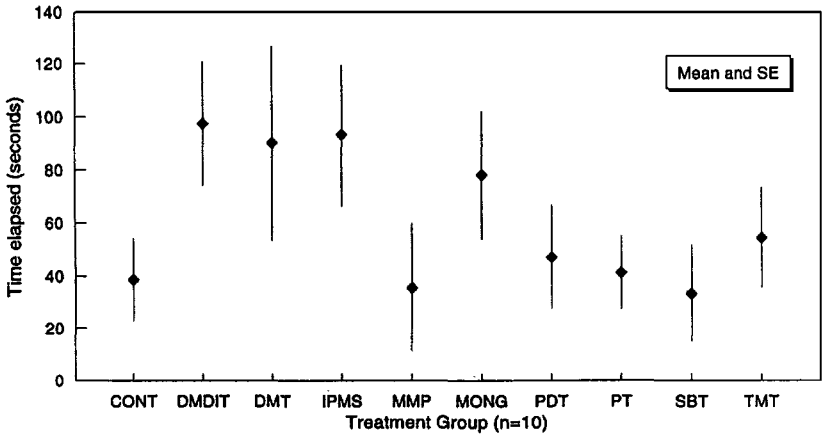


FIG. 2. Time elapsed (seconds) to initial arena entry for each of nine odor treatment groups and control (cont). Data are displayed with sex grouped. Each value is the mean ± SE.

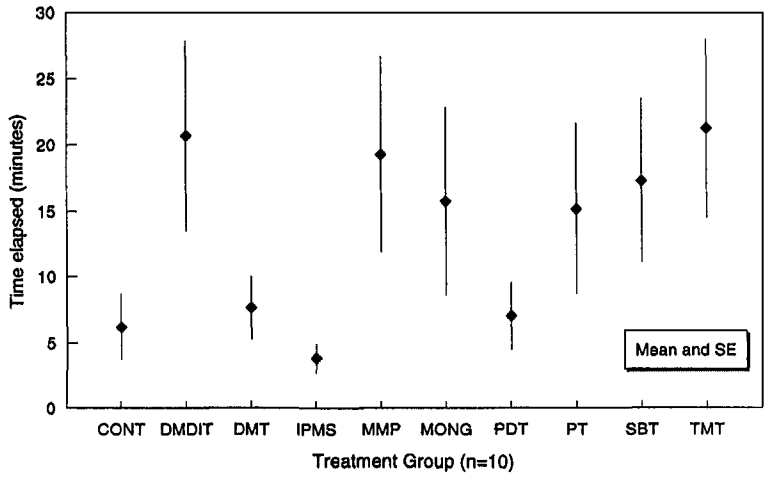


FIG. 3. Time elapsed (minutes) to first observed eating bout for each of nine odor treatment groups and control. Data are displayed with sex grouped. Each value is the mean ± SE.

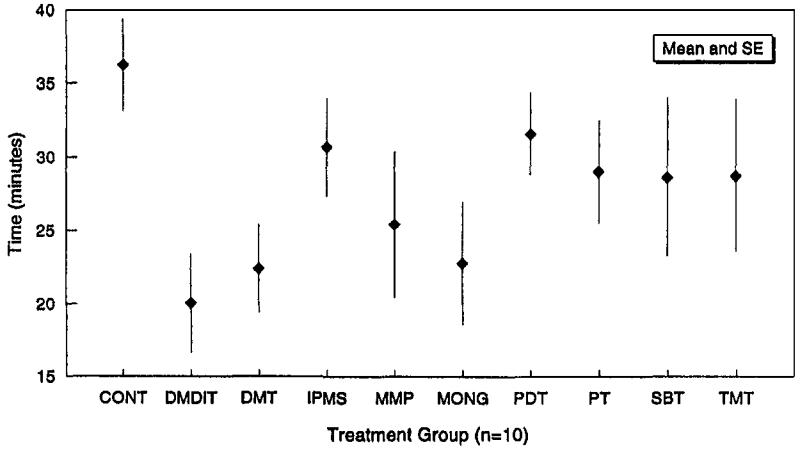


FIG. 4. Time spent (minutes) in arena (over 60 min) for each of nine odor treatment groups and control. Data are displayed with sex and sides grouped. Each value is the mean ± SE.

(22.4 ± 3.0 min), MMP (25.4 ± 5.0 min), and MONG (22.8 ± 4.2 min) were lower than that for control group rats (36.2 ± 3.1 min).

For the observed number of eating bouts there was a significant overall treatment effect (Figure 5). Duncan's multiple comparison test indicated that rats exposed to DMDIT or TMT had fewer ($P = 0.05$) eating bouts than rats exposed to either IPMS or PDT. Neither result was different from rats in the control group. There were no sex or side differences, and these results were grouped by treatment.

There were no side or treatment differences in food consumption. However, there was a significant difference in consumption by sex. Overall, mean consumption by male rats was 0.64 g and consumption by females was 0.41 g ($P = 0.03$). The data were grouped separately for each sex and displayed in Figures 6 and 7.

Summary of Trends in Results. Individual variability may have masked any differences due to the odors, and the sample size used was not great enough to identify differences statistically. By comparing the descriptive results summarized in Table 3, different trends across the measured variables for particular treatments become apparent. We based these trends on the probability values from statistical testing as well as the graphical representation of the results (Figures 2–7). From this summary, we were able to decide which odors produced the greatest potential behavioral avoidance response. We concluded that DMDIT, TMT, MMP, and MONG treatments had the most inhibitory effect on the behavior of roof rats.

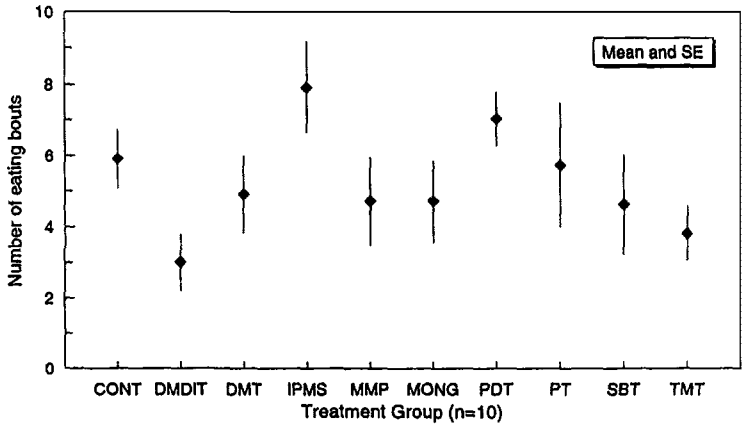


FIG. 5. Total number of eating bouts observed (over 60 min) for each of nine odor treatment groups and control. Data are displayed with sex and side grouped. Each value is the mean ± SE.

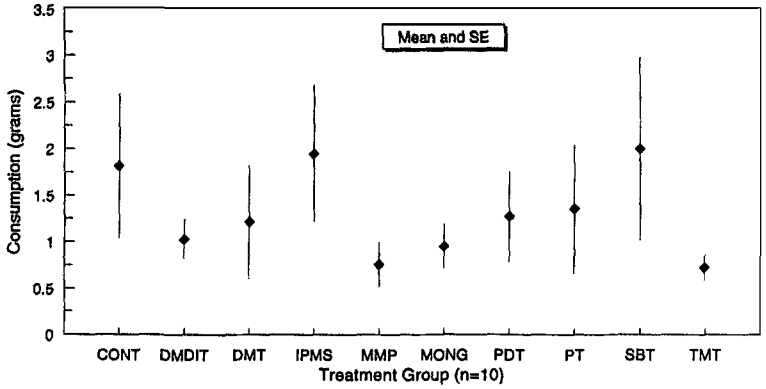


FIG. 6. Consumption (grams) by male rats for each of nine odor treatment groups and control. Data are displayed by side (treatment/control). Each value is the mean ± SE.

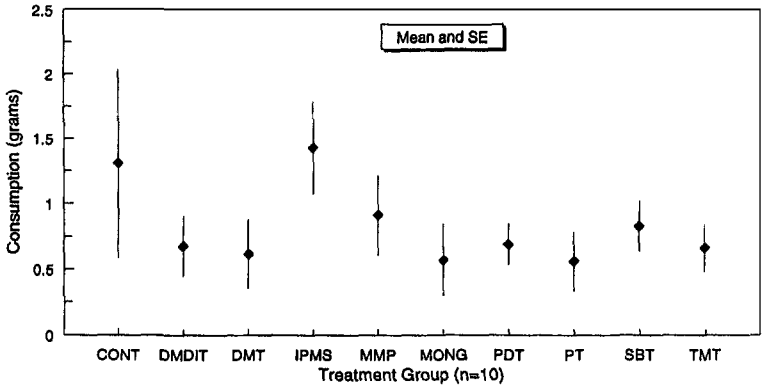


FIG. 7. Consumption (grams) by female rats for each of nine odor treatment groups and control. Data are displayed by side (treatment/control). Each value is the mean ± SE.

DISCUSSION

The intent of this study was to detect behavioral responses of rats to the presence of predator odors. The laboratory setting allowed for the control of environmental factors that could contribute to increased experimental error and reduced power to detect a response to the predator odors. Assessment of an avoidance response was developed based on criteria utilized in previous laboratory odor studies.

TABLE 3. SUMMARY OF TRENDS IN ROOF RAT RESPONSE TO TREATMENTS RELATIVE TO CONTROL GROUP RESPONSE[a]

Observation	Odor								
	DMDIT	DMT	IPMS	MMP	MONG	PDT	PT	SBT	TMT
Increased time to first entry ($P = 0.13$)	×	+	×	−	+	−	−	−	−
Increased time to first eating bout ($P = 0.19$)	×	−	−	×	+	−	+	×	×
Less time spent in arena ($P = 0.16$)	×	×	+	×	×	+	×	+	+
Lower number of eating bouts ($P = 0.05$, no differences from control	×	−	−	−	−	−	−	−	×
Lower consumption—males ($P = 0.03$, sex difference)	−	−	−	×	−	−	−	−	×
Lower consumption—females ($P = 0.03$, sex difference)	−	−	−	−	−	−	−	−	−

[a]Based on P values and descriptive results of Figures 2–7; X = difference; + = slight difference; − = similar results.

Many odor studies involving small rodents take place in a controlled laboratory setting, where behavioral responses can be observed directly. Some studies have combined the observed behavioral information with physiological measures such as changes in plasma hormone levels (Vernet-Maury et al., 1984). Other studies have focused on more quantitative responses to odors such as the amount of food consumed. Mountain beaver (*Aplodontia rufa*) showed reduced consumption from bowls scented with coyote (*Canis latrans*) urine compared with control bowls (Epple et al., 1993). Avoidance response of pocket gophers to stoat scent was determined by number of captures betwen scented and unscented traps (Sullivan and Crump, 1986). Chance and Mead (1955) showed that a greater stimulus change produced greater delays before a lab rat would start eating.

Previous laboratory odor studies have attributed fear responses to particular observed behaviors. However, there are often discrepancies in the interpretation of particular behavioral responses, and various researchers have attributed different meanings to similar activities. The observed behavior of defecation has been identified as a fear response (Vernet-Maury et al., 1984) and also as a nonfear response. Grooming and jumping are also two behaviors that are difficult to qualify as a stress response. Grooming observed in the prsence of predator odors has been described as a defensive response. Others have considered grooming to occur once the animal considers the surroundings safe (Vernet-Maury et al., 1984). Jumping is a common activity for roof rats and is often considered an exploratory behavior, especially in novel surroundings. Many of the common behavioral observations such as stretch, sniff, and rearing also appear to be more exploratory in nature and not necessarily indicative of a phobic response. Because of ambiguous interpretation of these behaviors, they were not used in this study. However, food consumption has been tested as a measure of avoidance of predator odors (Calder and Gorman, 1991; Epple et al., 1993; Heale and Vanderwolf, 1994). In our study, food consumption and other discrete observations were used to conclude whether or not the predator odors tested produced the desired response.

There are a wide range of responses one could potentially observe and interpret, and this study focused more on quantitative measurements. There is no preferred standard procedure to test the effect of predator odors, and hence an arena was designed to allow the observation of many likely behaviors. We also wanted to allow the individual a safe zone provided by access to a covered transfer cage. This design provided the individual with cover, food, and an exploration area, while its behavioral responses were observed in a two-choice situation. We assumed that if a particular odor signaled danger to a rat, then it would try to avoid the odor by delaying entry into the odor-treated arena, delaying time to first eating bout, spending less time on the treated side, and eating less from the treated side of the arena.

The results from the statistical analysis indicated no differences in the observed responses to predator odor treatments. We feel that much of the potential odor effects may have been hidden by the high individual variability observed in these rats. This variability in roof rats probably increased experimental error and reduced the power of statistical tests to detect significant results. This known variability may provide the rationale for raising α levels to increase the power of the test, while recognizing the elevated potential for type I errors. Recognition of this issue as well as the near significant statistical results ($P \approx 0.15$, see Table 3), suggested further consideration of the data.

The roof rat is considered a generalist species able to adapt readily to changes in the environment. This flexibility is partly due to the high variability in individual behavior. During the preliminary trials, differences in the response to the novel arena were observed. Some individuals spent the majority of time in the covered transfer cage, others ventured into the arena very cautiously, while some darted quickly between the two sides and the transfer cage. This variation between individuals has been suggested to explain *Rattus* species' adaptability (Ewer, 1971). Having a broad range of individual behavior responses allows the population as a whole to adapt readily to changes in the environment.

Other differences in responses may have occurred depending upon the type of predator odor tested. Jedrzejewski et al. (1993) showed different patterns of behavioral response of bank voles (*Clethrionomys glareolus*) to seven different predator odors. They found that voles responded differently to various species of carnivores and summarized these differences in a behavioral response table. Vernet-Maury et al. (1984) compared responses to predator odors using a behavioral score, based on whether the observation was considered that of a stress response or not. This technique allowed the combination of responses to give a final behavioral score with which to compare the odors. We followed a similar approach in combining the different response variables into a summary table (Table 3) to assist in interpreting the results.

The odors of interest were DMDIT, TMT, and MMP, all unfamiliar (in recent evolutionary times) synthetic odors, as well as MONG, a predator odor familiar to the roof rat. DMDIT delayed the time spent exploring/searching in the arena, increased the time elapsed to first eating bout, and reduced the time spent in the arena, the number of eating bouts, and the total consumption. MONG had similar results, although the total number of eating bouts was not different from the control group. TMT produced the greatest fear response in previous laboratory studies with rats (Vernet-Maury et al., 1984); this latter study also found that MMP and DMDIT produced a fear response, supporting the descriptive trends in this study.

Although no trials were performed to test for habituation, the results indicate that the roof rat tends to avoid mongoose feces, a familiar predator on most of the Hawaiian islands. The test rats had probably encountered mongoose odor

before being captured, and their apparent avoidance responses in the arena suggested that this fecal odor was recognized. This avoidance in the testing arena provided some evidence that the wild roof rat has not habituated to mongoose odor. These indications of avoidance in the testing arena may also reflect what has been demonstrated in the field. A recent live-trap study found lower capture success of root rats in traps that had previously captured a mongoose (Tobin et al., 1995).

The trends in avoidance of some of the synthetic odors tested resembled those for the mongoose odor. Based on the live-trap study of Tobin et al. (1995), it is expected that the synthetic predator odors would produce a similar avoidance response in the field. Although the synthetic odors lack any positive reinforcement in the field, we believe that an avoidance response will be observed initially. This prediction is supported by studies that indicate rodents have an innate recognition of predator odors. Cattarelli and Chanel (1979) demonstrated the greatest olfactory "awakening influence" for lab-reared Wistar rats occurred with red fox fecal odor. Vernet-Maury et al. (1984, 1992) have also demonstrated a fear response in lab rats when exposed to various predator odor chemicals. Orkney voles (*Microtus arvalis*), which have not been in contact with predators for generations also displayed an avoidance response when exposed to carnivore odors (Gorman, 1984). Whether this is a response to a particular common semiochemical is unknown, but definite behavioral responses have been observed in rodents exposed to various predator odors. The responses measured in the laboratory suggest an avoidance behavior by the wild Hawaiian roof rat to synthetic predator odors and support efforts to further test the odors in field trials.

CONCLUSIONS

Our laboratory bioassays indicated that responses to a predator odor could be detected by combining the results from various measures of observed behaviors. This descriptive summary approach has been used to combine results from various types of measurements (i.e., behavioral observations) and utilizes nonparametric analysis (Vernet-Maury et al., 1984). In the odor-testing arena, roof rats were observed to delay before entering, delay from first eating bout, spend less time in the arena, show a lower number of eating bouts, and consume less food in the presence of DMDIT odor. Mongoose fecal odor also produced similar avoidance responses, although they were not statistically significant. TMT and MMP synthetic predator odors also had avoidance trends in roof rats. These results support other laboratory findings in which avoidance or fear responses were recorded in rodents while in the presence of predator odors. A study with the same laboratory arena with modifications to the design and pro-

cedure found no difference in variables measured while testing mongoose odor treatments (Tobin et al., 1997). The nonsignificant results agree with the findings in our study. However, different variables were measured over a different time period, which may have contributed to a lack of observed descriptive trends. This same study (Tobin et al., 1997) also found lower capture rates in live-traps soiled with mongoose feces in the field portion of the study.

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SUPPRESSIVE IMPACT OF GLUCOSINOLATES IN *Brassica* VEGETATIVE TISSUES ON ROOT LESION NEMATODE *Pratylenchus neglectus*

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Abstract—The potential of tissue amendments from a variety of wild and cultivated *Brassica* spp. to kill the root lesion nematode (*Pratylenchus neglectus*) in soil was assessed in laboratory experiments. Soil amended with leaf tissues was highly nematicidal, killing 56.2–95.2% of exposed nematodes. Amendment with root tissues was less effective, causing 0–48.3% mortality. Little of the suppressive impact by leaf tissues could be related to either total contents or any individual glucosinolate as determined by HPLC analysis. While the levels of total glucosinolates within root amendments had a nonsignificant relationship to nematode mortality, levels of 2-phenylethyl glucosinolate within root amendments significantly correlated with nematode suppression ($P < 0.001$). Amendment of soils with equimolar levels of purified 2-phenylethyl isothiocyanate resulted in comparable levels of nematode mortality, suggesting that 2-phenylethyl glucosinolate has a role in the suppressive impact of *Brassica* spp. root tissues.

Key Words—*Brassica*, *Pratylenchus neglectus*, nematode, biological control, suppression, glucosinolate, isothiocyanate, aliphatic, 2-phenylethyl, nematicide.

INTRODUCTION

The inclusion of canola (*Brassica napus* L.) in Australian cereal rotations has increased in recent years due to improved crop adaptation, increased security of markets, and increasing awareness of its effectiveness as a disease break within

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the rotation (Potter, 1995). Canola rotations have been associated with reductions in numbers of harmful soil-borne organisms, resulting in earlier crop establishment, increased seedling vigor, and increased yields of succeeding wheat crops (Kirkegaard et al., 1994).

Glucosinolates are a group of related thioglycosides that occur naturally within the tissues of *Brassica* spp. (Fenwick et al., 1994). They are derived from amino acids and are stored in the vacuoles of cells of all tissue types within the plant. Over 100 different glucosinolates have been identified, belonging to aliphatic, indolyl, and aralkyl classes depending upon the chemical structure of their side chains (Underhill, 1980). Glucosinolates are considered to have little biological activity themselves but to be associated with plant defense (Fenwick et al., 1994). After tissue damage, these molecules are cleaved by a thioglucosidase (myrosinase; EC 3.2.1.1), producing many products including isothiocyanates, nitriles, and thiocyanates. Isothiocyanates are biologically active, disrupting cellular components by denaturing protein structure (Kawakishi et al., 1983). The variable structure of the side chain confers a variety of chemical properties to the isothiocyanate, influencing parameters such as volatility, miscibility, and membrane transmissibility (Fenwick et al., 1994). Individual isothiocyanates also exhibit distinct properties in terms of their biological activities (Drobnica et al., 1967; Giamoustaris et al., 1994; Horakova, 1966; Mithen, 1992; Mojtahedi and Santo, 1996).

Brassica tissues and the products of glucosinolates found within suppress a wide variety of soil organisms, including fungi (Glenn et al., 1988; Johnson et al., 1992; Mayton et al., 1996; Somda et al., 1995), viruses (Spak et al., 1993), bacteria (Brabban and Edwards, 1995), and weed seeds (Brown and Morra, 1995). Reduction in the number of many soil-borne plant parasitic nematodes has also been observed, including such species as *Meloidogyne incognita*, *M. javanica* (Johnson et al., 1992), *M. chitwoodi* (Mojtahedi et al., 1993), and *Heterodera schachtii* (Thierfelder and Friedt, 1995). Green manuring with tissues from *Brassica* spp. suppressed populations of the root lesion nematode (*Pratylenchus penetrans*) (McFadden et al., 1992), and reductions in *P. neglectus* populations in the soil were observed after a canola rotation (Parker, 1994). However, despite these observed decreases in *Pratylenchus* numbers, cultivated canola is susceptible to this nematode, having levels of nematode infestation and reproduction similar to susceptible wheat lines (Bernard and Montgomery-Dee, 1993; Webb, 1990). Because *B. napus* is a good host, allowing nematodes to feed and reproduce, the decrease in nematode numbers after a canola crop must be due to some factor other than reduced multiplication during the growth of a nonhost species.

The nematicidal properties of isothiocyanates have been tested on a range of soil nematodes, including *H. schachtii* (Donkin et al., 1995) and *C. elegans* (Lazzeri et al., 1993). Mojtahedi and Santo (1996) observed the suppression of

M. chitwoodi and *P. penetrans* by isothiocyanates and reported that impact of isothiocyanates may vary between nematode genera. The nematicidal impact of isothiocyanates has led to their commercial development for use as soil fumigants (Ashely and Leigh, 1963).

We examined the nematicidal impact of soil amended with tissues from various *Brassica* species having different glucosinolate profiles. The objective was to determine the relationship between glucosinolate content and the nematicidal impact of root and leaf tissues, emphasizing the contribution of specific glucosinolate products to nematode suppression.

METHODS AND MATERIALS

Source of Pathogens. P. neglectus nematodes were obtained from sterile carrot cultures, as described by Nicol and Vanstone (1993). Carrots were flooded with water, and the emerging nematodes were collected by filtration and suspended in sterile water at the desired density.

Source and Collection of Tissue. In order to maximize variation in leaf and root glucosinolate profiles, six accessions were selected from diverse genetic backgrounds within the *Brassica* genus. All seed was obtained from the Victorian Institute for Dryland Agriculture, Horsham, Victoria, Australia, except for the *Brassica juncea* line, which was supplied by Dr. Rex Oram, CSIRO Division of Plant Industry, Canberra, Australia. The lines selected were *B. napus* cv. Dunkeld acc. no. 94713, *B. oxyrrhina* acc. no. 95060, *B. nigra* acc. no. 95067, *B. campestris* acc. no. 90139, *B. juncea* acc. no. 99Y11, and *B. carinata* acc. no. 94044. Seed was germinated on filter paper and sown in 125-mm pots containing a standard University of California (UC) soil mix. Plants were grown under ambient glasshouse conditions. Tissues of seven plants of each line were harvested at floral initiation. Roots were washed quickly to remove soil and placed in liquid nitrogen. Tissues were lyophilized, ground into a powder in a domestic coffee grinder, combined by line, and stored in a dessicator over silica gel until required for nematode and HPLC assays (Williams et al., 1993).

Suppression Trials. The method for testing the nematicidal impact of the *Brassica* tissues and/or pure isothiocyanates was adapted from conventional nematicide assay techniques (McBeth and Bergeson, 1953).

Tissue Amendment Studies. Desiccated tissues were mixed thoroughly into steam sterilized, oven dried UC soil at a level of 20 mg tissue per gram soil (2%). This rate of tissue amendment was selected following preliminary trials (unpublished data) that showed this level of soil amendment was sufficient to cause an average of 50% nematode mortality in a range of lines. Soil containing amendment tissue was weighed into a 70-ml screw top plastic vial and hydrated

by the addition of sterile water (1 ml for every 5 g soil). Because hydration allows release and escape of tissue isothiocyanates, 1 ml of an aqueous suspension with 500 nematode per milliliter was added quickly to the soil, and the vial was sealed and placed at room temperature in the dark for 72 hr.

Purified Isothiocyanate. 2-Phenylethyl isothiocyanate was obtained from Sigma chemicals (code: P-2179). A dilution series (15, 35, 50, 70, 85, 100 nmol/g soil) of the isothiocyanate was made in 0.5-ml aliquots of ethanol with 0.5 ml of ethanol as a control. Each aliquot was mixed thoroughly into 20 g of sterile, oven-dried UC soil in a 70-ml screw top vial that was then hydrated with 2.5 ml of sterile distilled water. To each vial was added 1 ml of a 200-nematode/ml aqueous suspension, and the vial was sealed immediately and placed at room temperature in the dark for 72 hr.

Retrieval of Nematodes. Surviving nematodes were extracted from the soil by a modified mister extraction technique (Southey, 1986). Soil was kept continuously wet in the mister apparatus for 94 hr. Nematodes retrieved from the flooded soil were concentrated, suspended in a known volume of water, and a 1-ml sample was taken for counting (Doncaster, 1962).

Glucosinolate Analyses. Glucosinolates were desulfated and assessed by HPLC as described by Heaney et al. (1986) using a Waters Lambda Max LC Spectrophotometer (model 481) at 229 nm and a Waters Spherisorb S5 ODS2 column (4.6 × 250 mm). Glucosinolates were identified relative to known standards and quantified with reference to an internal standard of 2-propenyl or benzyl glucosinolate (courtesy of Dr. Richard Mithen, Department of Brassica and Oilseeds Research, John Innes Centre, Norwich, UK).

Statistical Analyses of Results. Nematode suppression was calculated from numbers of nematodes retrieved from amended soils and expressed as a percentage of the control. Correlation between nematode suppression and tissue glucosinolates was undertaken by analysis of variance with the Genstat statistical package, and significance was assessed at the 5% level. Fitted curves on figures were produced by regression analysis.

RESULTS AND DISCUSSION

Suppression of P. neglectus *by Tissue Amendment.* Amendments of soil by *Brassica* spp. leaf and root tissues significantly reduced populations of *P. neglectus* nematodes (Table 1, rows 1 and 2). Leaf tissue amendments had a greater suppressive impact than otherwise identical quantities of root tissues from the same plants. The weed accession, *B. oxyrrhina*, was particularly effective, as leaf and root tissue amendments suppressed 95% and 48% of nematode populations, respectively. Both root and leaf tissues of the commercially grown canola line (*B. napus* var. Dunkeld) also significantly suppressed nematodes in

TABLE 1. VARIATION IN NEMATICIDAL IMPACT OF *Brassica* spp. LEAF AND ROOT TISSUES AGAINST *Pratylenchus neglectus*[a]

	Nematode suppression (% of population killed)[b]											
	B. napus		*B. oxyrrhina*		*B. rapa*		*B. nigra*		*B. carinata*		*B. juncea*	
Leaf	57.2	(11.9)[c]	95.2	(2.5)[c]	66.2	(10.8)[c]	56.2	(12.1)[c]	65.2	(9.6)[c]	68.2	(6.2)[c]
Root	31.7	(7.7)[c]	48.3	(15.9)[c]	<0	(6.5)	<0	(7.8)	<0	(14.3)	13.5	(12.7)[c]
Leaf[d]	<0	(11.9)	32.6	(2.5)[c]	3.6	(10.8)	<0	(12.1)	<0	(9.6)	5.6	(6.2)

[a]Data displayed are relative to the suppressive impact of wheat tissues.
[b]Mean where $N = 7$, $\pm$ standard error of the mean in parentheses.
[c]Significantly different to 0% suppression at the 0.05 level.
[d]Suppression above the nil glucosinolate level. (Calculated by subtracting 'neglible glucosinolate' leaf suppression from 'total' leaf suppression).

the soil. This finding supports reports from the field that degraded canola tissues may cause reductions in root diseases, increasing the fitness of the soil environment for subsequent crops (Kirkegaard et al., 1994).

Leaf Amendments and Their Glucosinolates. The glucosinolate profiles within the leaf tissues were dominated by aliphatic glucosinolates (Table 2). The leaf tissues of the *B. nigra*, *B. juncea*, and *B. carinata* lines contained high levels of 2-propenyl, contributing almost 100% of the glucosinolate profile. While leaves from the *B. oxyrrhina* line also contained high total levels of aliphatic glucosinolates, these tissues contained 3-butenyl and 4-pentenyl (rather than 2-propenyl) glucosinolates. Leaf tissues from the *B. napus* and *B. rapa* lines contained low levels of aliphatic glucosinolates, and consequently low total levels. Indolyl and aralkyl glucosinolates were also present at trace levels within the *B. napus*, *B. rapa*, and *B. oxyrrhina* leaves.

No significant relationship was observed between total amendment glucosinolate levels and observed nematode suppression (Figure 1A). However, there appears to be a base level of suppression ($\sim 60\%$) regardless of the glucosinolate content in the tissues (Figure 1A) as both *B. napus* and *B. rapa* leaf tissues caused more than 55% mortality (Table 1), despite the fact that their amendments gave rise to less than 10 nmol glucosinolate per gram soil (Table 2). It seems likely that other chemicals, independent of the glucosinolate allelopathic system, contributed to the suppressive impact of the leaf tissues. Many biologically active organic chemicals, including alkenals and alkanols, are released from senescent tissues (Bradow, 1991; Jiang et al., 1996) and may contribute to the nematicidal impact of *Brassica* leaf tissues.

To assess the actual impact of the glucosinolates within the leaf tissue, the suppression caused by *B. napus* and *B. rapa* tissue (containing negligible levels of glucosinolate) was considered reflective of nonglucosinolate suppression and was subtracted from the total suppression observed (Table 1, row 3). No significant relationship was observed between leaf suppression and total glucosinolates (Figure 1A) or total aliphatic glucosinolates (Figure 2A).

The glucosinolates in leaf tissue were only observed to impart a significant increase in suppression in the case of the *B. oxyrrhina* leaf tissues. It is possible that the particularly high total levels of glucosinolate within this tissue surpassed a critical level (above 200 nmol/g soil; Figure 1A), after which sufficient isothiocyanate was liberated to impact the nematode population. As the glucosinolate profile only within this tissue imparted a significant effect, it was difficult to draw any reliable correlation between nematode suppression and individual glucosinolates. However, the presence of high levels of 3-butenyl and 4-pentenyl glucosinolates within the *B. oxyrrhina* amendment (imparting soil levels of 166.76 and 33.92 nmol/g soil, respectively; Table 2) seems likely to be responsible for the observed suppression above the base level. This is supported by

TABLE 2. LEVELS OF GLUCOSINOLATE IN VARIOUS *Brassica* LEAF AND ROOT TISSUES AND LEVELS OF GLUCOSINOLATES PER GRAM SOIL AFTER AMENDMENT WITH 0.2 g OF TISSUE

Glucosinolate	Class	Glucosinolate level [μmol/g tissue (nmol/g soil)]					
		B. napus	*B. oxyrhina*	*B. rapa*	*B. nigra*	*B. juncea*	*B. carinata*
Leaf							
2-Hydroxy-3-butenyl	aliphatic	0.38 (1.52)	0	0.52 (2.08)	0	0	0
2-Prophenyl	aliphatic	0	0	0	25.18 (100.72)	23.68 (94.72)	40.52 (162.08)
4-Methyl sulphonyl butyl	aliphatic	0	0.43 (1.72)	0.15 (0.60)	0	0	0
3-Butenyl	aliphatic	0.10 (0.40)	41.69 (166.76)	0.1 (0.40)	0	0	0
4-Pentenyl	aliphatic	0.19 (0.76)	8.48 (33.92)	1.59 (6.36)	0	0	0
3-Indolylmethyl	indolyl	0.99 (3.96)	0.65 (2.60)	0.31 (1.24)	0	0	0.37 (1.48)
2-Phenylethyl	aralkyl	0.12 (0.48)	0.64 (2.56)	0.21 (0.84)	0	0	0
4-Methoxy-3-indolylmethyl	indolyl	0.14 (0.56)	0.4 (1.16)	0.4 (1.60)	0	0	0
1-Methoxy-3-indolylmethyl	indolyl	0.27 (1.08)	0.18 (0.72)	0.05 (0.20)	0	0	0
Σ aliphatic glucosinolates		0.67 (2.68)	50.61 (202.44)	2.35 (9.4)	25.18 (100.72)	23.68 (94.72)	40.52 (162.08)
Σ glucosinolate		2.17 (6.68)	52.36 (209.44)	3.32 (13.28)	25.18 (100.72)	23.68 (94.72)	40.89 (163.56)
Root							
2-Propenyl	aliphatic	0	0	0	3.08 (12.32)	6.68 (26.72)	1.64 (6.56)
3-Butenyl	aliphatic	0	0.63 (2.52)	0	0	0	0
4-Pentenyl	aliphatic	0	0.63 (2.52)	0.14 (0.56)	0	0	0
2-Phenylethyl	aralkyl	12.73 (50.92)	14.41 (57.64)	3.00 (12.00)	4.45 (17.80)	10.42 (41.68)	0.87 (3.48)
Σ aliphatic glucosinolates		0	1.26 (5.04)	0.14 (0.56)	3.08 (12.32)	6.68 (26.72)	1.64 (6.56)
Σ glucosinolate		12.73 (50.92)	15.67 (62.68)	3.14 (12.56)	7.53 (30.12)	17.1 (68.40)	2.51 (10.04)

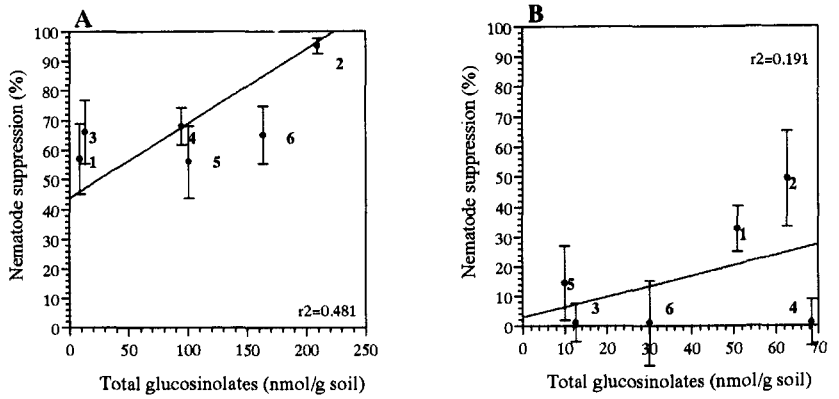

FIG. 1. Relationship between total glucosinolates in amendments and nematode suppression (expressed as % relative to wheat tissue control). (A) Leaf tissue; (B) root tissue. 1 = *B. napus*, 2 = *B. oxyrrhina*, 3 = *B. rapa*, 4 = *B. nigra*, 5 = *B. juncea*, 6 = *B. carinata* (mean ± SE; $N = 7$).

the significant suppression of *P. penetrans* by soil amendment with purified 3-butenyl isothiocyanate (Mojtahedi and Santo, 1996). Amendments with *B. nigra*, *B. juncea*, and *B. carinata* tissues did not significantly increase nematode suppression above the base level despite the high levels of 2-propenyl glucosinolate within the tissue (conferring over 160 nmol glucosinolate per gram soil

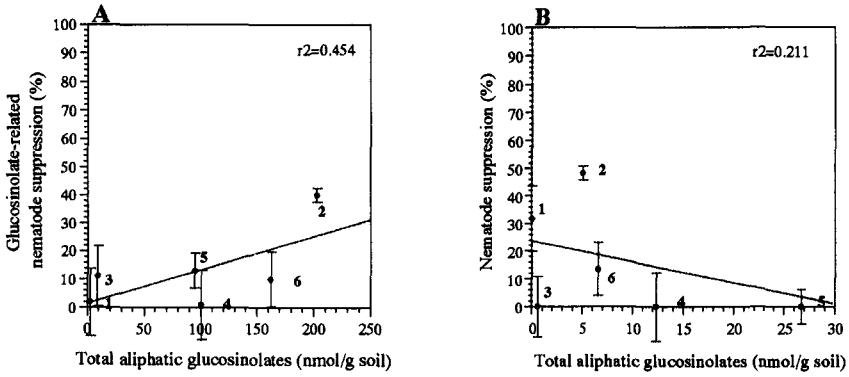

FIG. 2. Relationship between total aliphatic glucosinolates in amendment tissues and observed nematode suppression (expressed as % of wheat leaf tissue). (A) Leaf; (B) root. 1 = *B. napus*, 2 = *B. oxyrrhina*, 3 = *B. rapa*, 4 = *B. nigra*, 5 = *B. juncea*, 6 = *B. carinata* (mean ± SE; $N = 7$).

in the case of *B. carinata* leaf amendment; Table 2). This result is in contrast with findings of Mojtahedi and Santo (1996), who suggested that soils amended with 2-propenyl isothiocyanate exhibited a greater nematode suppression than those amended with 3-butenyl isothiocyanate. Mayton et al. (1996) and Williams et al. (1993) also reported the high biocidal activity of 2-propenyl isothiocyanate. High levels of 2-propenyl glucosinolate were observed in amended leaf tissues (Table 2), and it may be that liberation of the isothiocyanate from the glucosinolate was inhibited in some way. Alternatively, the isothiocyanate released may not have been effective in the environment produced in the trial.

Root Amendments and Their Glucosinolates. Analyses of root tissues showed lower total glucosinolate levels than observed within leaf tissues from the same plants (Table 2). Low levels of aliphatics and high levels of the aralkyl glucosinolate, 2-phenylethyl were observed (Table 2). This supports previous findings that much of the root glucosinolate profile of *Brassica* spp. consists of the aralkyl class (Sang et al., 1984). Root tissues from *Brassica napus* and *B. oxyrrhina* lines contained almost exclusively high levels of 2-phenylethyl glucosinolate relative to the other lines studied. Roots from the *B. juncea* accession contained moderate levels of 2-phenylethyl and a small amount of 2-propenyl glucosinolate. Root tissues from the *Brassica rapa*, *B. nigra*, and *B. carinata* accessions contained comparatively low levels of glucosinolates, with more aliphatic than aralkyl glucosinolates.

Although there is an apparent positive trend, no significant relationship between total root glucosinolate levels and nematode suppression was observed (Figure 1B).

Although no relationship was observed between suppression and the aliphatic glucosinolates present in the roots (Figure 2B), levels of 2-phenylethyl glucosinolate correlated significantly ($P < 0.001$) with observed nematode suppression (Figure 3). Suppression was observed at levels lower than 45 nmol/g soil (*B. juncea*) and reached levels in excess of 45% in the case of the *B. oxyrrhina* amendment (conferring >55 nmol/g soil; Table 2). As glucosinolates themselves are not nematicidal, acting only to liberate toxic molecules following tissue damage (Fenwick et al., 1994), the relationship between suppression and amendment levels of 2-phenylethyl glucosinolate suggests that a breakdown product of this compound may have been at least partially responsible for the observed nematode suppression. Consequently, we investigated the nematicidal impact of purified 2-phenylethyl isothiocyanate.

Nematicidal Impact of Pure 2-Phenylethyl Isothiocyanate. Purified 2-phenylethyl isothiocyanate was added to soils at levels similar to those expected to be liberated from complete hydrolysis of 2-phenylethyl glucosinolate from root tissue amendments in previous studies (Figure 3). These amendments were significantly ($P < 0.05$) related to nematode suppression (Figure 4), confirming nematicidal impact of this molecule.

Nematode suppression was comparable after amendment with similar levels of 2-phenylethyl glucosinolate (within root tissue; Figure 3) and 2-phenylethyl isothiocyanate (in purified form; Figure 4), suggesting that the suppression by root tissue amendments was due to liberation of 2-phenylethyl isothiocyanate from the glucosinolate following rehydration of tissue in the soil. However, the linear relationship between 2-phenylethyl isothiocyanate and nematode suppression (Figure 4) was in contrast to the exponential relationship observed between suppression and root amendment levels of 2-phenylethyl glucosinolate (Figure 3). The lag in suppressive activity associated with the tissue studies suggests that a critical level of glucosinolate was required before suppression occurred. As isothiocyanate release after glucosinolate hydrolysis is dependent upon many factors (Fenwick et al., 1994), the differences between the soil environments within the assays under comparison may be responsible for differences in response. Components of plant tissue present in the tissue amended bioassays but absent in the purified isothiocyanate studies, may have bound or deactivated the active isothiocyanate as it was released, perhaps explaining the immediate linear relationship observed between chemical amendment levels and nematode suppression (Figure 4).

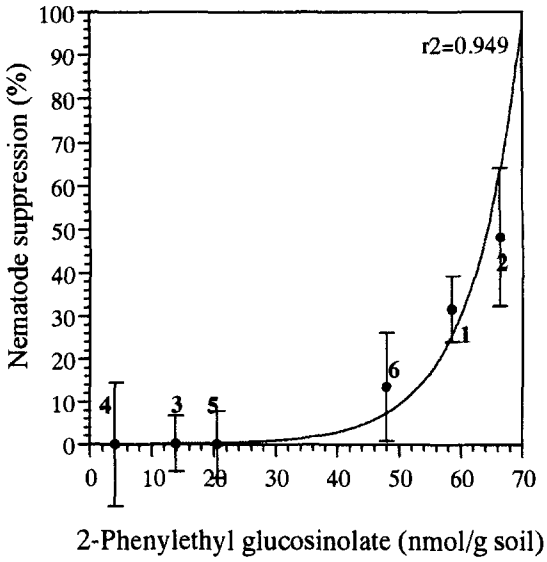

FIG. 3. Relationship between root and leaf amendment levels of 2-phenylethyl glucosinolate and observed nematode suppression (expressed as % of wheat tissue control). 1 = *B. napus*, 2 = *B. oxyrrhina*, 3 = *B. rapa*, 4 = *B. carinata*, 5 = *B. nigra*, 6 = *B. juncea* (mean ± SE; $N = 7$).

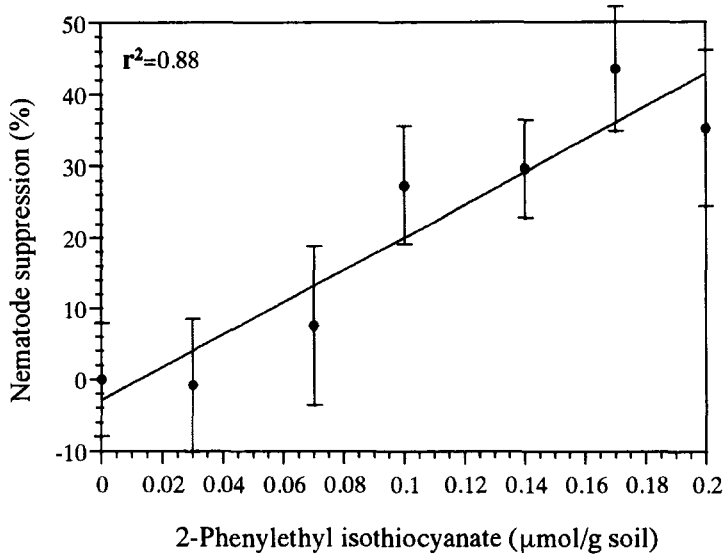

FIG. 4. Impact of purified 2-phenylethyl isothiocyanate upon nematode suppression in the soil. (mean ± SE; $N = 7$).

CONCLUSIONS

Incorporation of *Brassica* leaf and root tissues into soil containing *P. neglectus* caused a significant reduction in nematode numbers when compared to soil amended with wheat tissues. The data support past findings that *Brassica* tissues actively suppress populations of *Pratylenchus* (McFadden et al., 1992). The significant relationship between suppression by root tissues and the levels of 2-phenylethyl glucosinolate within *Brassica* tissue suggests that the suppression is dependent upon the presence of specific glucosinolates, rather than the total levels present. This is supported by the finding that soils amended with tissue containing similar total levels of glucosinolates (~65 nmol/g soil; *B. oxyrrhina* and *B. juncea* roots) exhibited significantly different levels of nematode suppression (48.3% and 13.5%, respectively). It is also evidence that the complement of glucosinolates within plant tissues can influence host–pathogen interactions of that plant tissue (Giamoustaris et al., 1994; Mithen, 1992) and supports findings that nematicidal potency of individual isothiocyanates may be influenced by the nature of their side chains (Drobnica et al., 1967; Horakova 1966; Mojtahedi and Santo, 1996).

The biological activity of aliphatic isothiocyanates against soil-borne organisms has been widely discussed (Lazzeri et al., 1993; Brabban and Edwards,

1995; Williams et al., 1993), and methyl isothiocyanate (also aliphatic) is utilized as a commercial nematicide (Ashley and Leigh, 1963). Actual isothiocyanate levels liberated from tissue amendments were not assessed here, and it is therefore difficult to draw conclusions about isothiocyanate activity merely from correlation with aliphatic glucosinolate levels (particularly with different tissue types such as leaves and roots). However, our results suggest that amendments containing high levels of aliphatic glucosinolates are not effective in the suppression of *P. neglectus*, only significantly correlating when glucosinolate levels were very high (>200 nmol/g soil). In contrast, the levels of 2-phenylethyl glucosinolate in amendments were significantly correlated with nematode suppression at levels below 70 nmol/g soil. Aliphatic isothiocyanates, although active in vitro, may not act efficiently in the soil environment due to their chemical nature (as supported by Williams et al., 1993, and Matthiesson et al., 1996).

The biocidal impact of 2-phenylethyl isothiocyanate has been reported previously. Horakova (1966) observed that 2-phenylethyl isothiocyanate was active against HeLa cells, exhibiting more than three times the toxicity of 2-propenyl isothiocyanate and an ED_{50} similar to that of methyl isothiocyanate. It was also reported to have 30 times greater the antifungal potency than 2-propenyl isothiocyanate against *Aspergillus niger*, *Penicillium cyclopium*, and *Rhizopus oryzae* (Drobnica et al., 1967). The structure of the molecule suggests that it may be effective in the soil environment as it is less volatile than the smaller aliphatic type isothiocyanates. The hydrophobic phenyl group may provide stability within the soil, lodging within soil organic matter, while the ethyl tail may act to hold the isothiocyanate group free to make contact with soil organisms. Mithen et al. (1995) suggested that glucosinolates producing isothiocyanates exhibiting physiologically varied activities may be responsive to selection pressures, leading to the development of a tissue profile best suited to the environment in which the isothiocyanate is likely to be released. As 2-phenylethyl glucosinolate accumulates almost to the exclusion of all others within the root tissues of *Brassica* spp. (Sang et al., 1984), it would not be surprising to find that 2-phenylethyl isothiocyanate is efficient in the soil environment.

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INSECTICIDAL AND ACARICIDAL ACTIVITY OF CARVACROL AND β-THUJAPLICINE DERIVED FROM *Thujopsis dolabrata* var. *hondai* SAWDUST

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Abstract—Insecticidal and acaricidal components from sawdust of *Thujopsis dolabrata* var. *hondai* against eight species of anthropod pests (*Reticulitermes speratus*, *Lasioderma serricorne*, *Callosobruchus chinensis*, *Sitophilus oryzae*, *Plutella xylostella*, *Myzus persicae*, *Blatella germanica*, and *Tetranychus urticae*) were isolated by chromatographic techniques and characterized by spectroscopic analyses as the terpenoids carvacrol and β-thujaplicine. In tests using the filter paper diffusion method, carvacrol had broad insecticidal and acaricidal activity against agricultural, stored-product, and medical arthropod pests. However, β-thujaplicine showed only weak termiticidal activity. Insecticidal activity of carvacrol was attributable to fumigant action. As a naturally occurring insecticide, carvacrol could be useful as a new preventive agent against damage caused by these arthropod pests.

Key Words—Natural insecticide, stored-product insects, *Thujopsis dolabrata* var. *hondai*, Cupressaceae, carvacrol, terpenoid, fumigation, β-thujaplicine.

INTRODUCTION

Insect pest control in the agricultural and medical fields continues to focus on the development and use of broad-spectrum synthetic insecticides. However,

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their continued or repeated use over several decades has disrupted control by natural enemies and has led to outbreaks and resurgences of pests (Ripper, 1956; McClure, 1977), the development of widespread resistance to insecticides (Georghiou and Saito, 1983; National Research Council, 1986), undesirable effects on nontarget organisms, environmental problems, and human health hazards (Brown, 1978). Besides these problems, factors such as labor and insecticide application costs should be considered. This economic consideration, along with decreased efficacy, and increasing concern over adverse effects of the earlier types of insecticides have brought about the need for the development of new types of selective control alternatives or biorational management methods without, or with reduced, use of synthetic insecticides.

Plants constitute a rich source of bioactive chemicals such as phenolics, terpenoids, and alkaloids (Harborne, 1993). Since these are often active against a limited number of species, including specific target insects, are biodegradable to nontoxic products, and are potentially suitable for use in integrated pest management programs, they could lead to the development of new classes of safer insect control agents. Therefore, much effort has been focused on plant materials for potentially useful products as commercial insecticides or as lead compounds (Jacobson and Crosby, 1971; Elliott, 1997; Hedin, 1982; Arnason et al., 1989a; Isman, 1995).

In laboratory and field observations, we found that *Thujopsis dolabrata* var. *hondai* Sieb. et Zucc. (Family Cupressaceae), the only Japanese *Thujopsis* species, is resistant to insect attack. It is interesting that a small pagoda (Nara, Japan) made from this tree was discovered in a well-preserved state without any damage by insects such as termites after almost 1200 years (Kawaguchi et al., 1989). We have, therefore, examined extracts of the sawdust from this tree for insecticidal and acaricidal constituents.

METHODS AND MATERIALS

Chemicals. Carvacrol and β-thujaplicine were purchased from Sigma (St. Louis, Missouri) and Extra Synthese (France), respectively. All other chemicals were of reagent grade.

Test Animals. Eight species of arthropod pests were used in this study. *Reticulitermes speratus* (Kolbe) nymphs, *Lasioderma serricorne* (Fab.) adults, *Callosobrushus chinensis* (L.) female adults, *Sitophilus oryzae* (L.) female adults, *Phutella xylostella* (L.) larvae (second to third instar), *Myzus persicae* (Sulzer) adults, *Blatella germanica* (L.) nymphs (second instar), and *Tetranychus urticae* (Koch) female adults. They were maintained in continuous culture through numerous generations without exposure to any insecticide.

Isolation and Identification. The steam distillate of *Thujopsis dolabrata*

var. *hondai* sawdust was obtained from the Central Research Laboratories, Taiyo Kagaku, Co., Yokkaichi, Mie, Japan. It (50 g) was fractionated by centrifugal thin-film evaporation as previously described (Ahn et al., 1995), and the fractions I (white color, 2.6 g), II (blue color, 18.1 g), III (red color, 12.3 g), and IV (black color, 17 g) were bioassayed against *R. speratus* and *B. germanica* nymphs. Fraction I (2 g) was chromtographed on a silica gel column (Merck, 230–400 mesh, 100 g, 5 × 100 cm), and components were eluted with hexane–ethyl acetate (10:1). Column fractions were analyzed by TLC (chloroform–methanol, 10:3), and fractions with similar TLC patterns were pooled.

Further separation of the biologically active components was carried out with HPLC (Hitachi 655A-11 LC). Develosil 60-5 (Nomura Chemical, Japan) and Cosmosil $_5C_{18}$ columns (Nacalai Tesque, Japan) were used with isocratic mobile phases consisting of hexane–ethyl acetate (9:1) and acetonitrile–water (10:1), respectively. Eluates were monitored at 254 nm. Two active components were isolated.

Structural determination of the active isolates were made by spectroscopic analyses. ^1H and ^{13}C NMR spectra were recorded with a Bruker AMX-500 spectrometer (TMS as an internal standard). UV spectra were obtained on a Hitachi 340 spectrophotometer, IR spectra on a Biorad FT-80 spectrophotometer, and mass spectra on a JEOL GSX 400 spectrometer.

Bioassay. A filter paper diffusion method was used to test the insecticidal and acaricidal properties of the isolates. Appropriate doses of the isolates in 100 μl of acetone were applied to filter papers (Whatman, 35 mm diameter). Each paper was placed in the bottom of a polyethylene cup (50 mm diameter × 35 mm), and test insects were placed in each cup with a lid.

The susceptibility of *R. speratus* nymphs and *S. oryzae* female adults to the steam distillate and its active isolates was investigated. Each filter paper treated with appropriate doses was placed in the bottom of the polyethylene cup. Each cup was packed with 40 g of sterile sea sand B (Nakalai, Japan), moistened with water, and then test arthropods were placed in each cup either with or without a lid.

All treated materials were held at 25 ± 1°C, 50–60% relative humidity, and 16L:8D. Mortality was determined 48 hr after treatment. Test animals were considered dead if appendages did not move when prodded with a camel's hair brush. LD_{20} values were determined by probit analysis (SAS, 1989). Controls received 100 μl of acetone, and mortality did not exceed 2.5%. Each assay was conducted in triplicate. Data from all bioassays were corrected for control mortality by using Abbott's (1925) formula.

Statistical Analysis. The percentage mortality was determined and transformed to arcsine values for analysis of variance (ANOVA). Treatment means were compared and separated by Scheffe's test at $P = 0.05$ (SAS, 1989). Means (± SE) of untransformed data are reported.

RESULTS

Identification. When the steam distillate from *T. dolabrata* sawdust was subjected to bioassay, we observed insecticidal activity against *R. speratus* and *B. germanica.* In further fractionation, the steam distillate was partitioned into four fractions by centrifugal thin-film evaporation. Fractions I and II showed strong and weak insecticidal activities, respectively (Table 1).

Purification of the biologically active components from fraction I (2 g) was accomplished by silica gel column chromatography and HPLC. Two active isolates among eight peaks showed insecticidal activity. Structural determination of the isolates was made by spectroscopic analyses and by direct comparison with authentic reference compounds (Ahn et al., 1995), and they were characterized as the terpenoids carvacrol (6.2 mg) and β-thujaplicine (12.2 mg) (Figure 1).

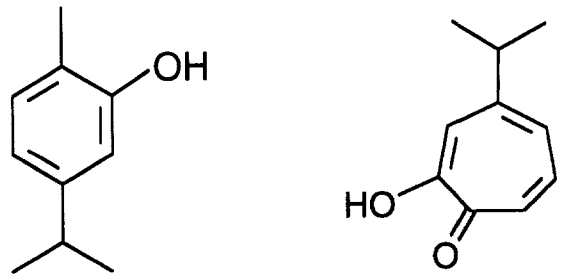
Insecticidal and Acaricidal Activity. The insecticidal and acaricidal spectrum of the isolates was assessed by a filter paper diffusion method (Table 2). Carvacrol has potent insecticidal and acaricidal activity against a range of agricultural, stored-product, and medical arthropod pests and is most active as a termiticide; β-thujaplicine, however, showed little or no insecticidal or acaricidal activity against the arthropods tested.

We were interested in determining whether the insecticidal activity of the steam distillate and carvacrol against *R. speratus* nymphs and *S. oryzae* female

TABLE 1. INSECTICIDAL ACTIVITY OF *Thujopsis dolabrata* var. *hondai* SAWDUST-DERIVED MATERIALS AGAINST *Reticulitermes speratus* NYMPHS AND *Blatella germanica* FEMALE ADULTS BY THE FILTER PAPER DIFFUSION METHOD

Test material	Mortality (mean ± SE); %)[a]			
	N	*R. speratus*[b]	*N*	*B. germanica*[a]
Crude oil	78	100a[c]	30	56.7 ± 6.7b[c]
Fraction				
I	84	100a	30	100a
II	84	56.0 ± 4.3b	30	53.3 ± 6.7b
III	75	0c	30	0c
IV	75	0c	30	0c

[a] Means within a column followed by the same letter are not significantly different ($P = 0.05$, Scheffe's test) (SAS, 1989). Mortalities were transformed to arcsine square root before ANOVA. Means (± SE) of untransformed data are reported.
[b] 1.5 mg/filter paper.
[c] 5.0 mg/filter paper.



Carvacrol **β-Thujaplicine**

FIG. 1. Structures of terpenoids isolated from *Thujopsis dolabrata* var. *hondai* sawdust.

adults was attributable to contact toxicity or fumigant action. Therefore, three different treatment methods were tested (Table 3). Potent insecticidal activity of the distillate was observed when cups were covered with lids compared to those without lids. Similar results were also obtained with carvacrol. Toxicity of carvacrol was enhanced in these insect species when they were permitted to contact the treated filter paper.

DISCUSSION

T. dolabrata belongs to the family Cupressaceae. Jacobson (1989) pointed out that the most promising sources of botanical insecticides for use at present and in the future are species of the families Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labiatae, and Canellaceae. Reported biological activities of plant terpenoids include repellency and deterrency, reduced palatability, growth inhibition through altered protein availability, enzyme inhibition, and direct toxicity (Harborne, 1993). Jointly or independently, these may contribute to the protection of plants against herbivores and pathogens, although some herbivores have counteradapted to terpenoids.

Many *Thujopsis* species are a rich source of terpenoids (Hasegawa and Hirose, 1982; Yatagai et al., 1985). Toxicological and pharmacological investigations have demonstrated that *Thujopsis* species have antimicrobial (Ito et al., 1980), hypocholesterolemic (Enomoto et al., 1977), insecticidal (Nakashima and Shimizu, 1972; Chaboussou, 1978; Lee et al., 1997), and rodent-repellent effects (Ahn et al., 1995). In our study, carvacrol showed potent insecticidal and acaricidal activity with a broad spectrum against a limited species of agricultural, stored-product, and medical arthropod pests. The compound was much more effective in closed cups than in open ones. Carvacrol has a characteristic odor and is highly volatile (Budavari et al., 1989). These results suggest that

TABLE 2. INSECTICIDAL AND ACARICIDAL SPECTRUM OF CARVACROL AND β-THUJAPLICINE BY FILTER PAPER DIFFUSION METHOD

Test insect	LD_{50} [(95% CL), mg/filter paper]			
	N	Carvacrol	*N*	β-Thujaplicine
Reticulitermes speratus (Isoptera: Rhinotermitidae)	240	0.27 (0.24–0.32)	240	24.61 (22.64–26.36)
Lasioderma serricorne (Coleoptera: Anobiidae)	240	1.47 (1.34–1.61)	120	>100
Callosobruchus chinensis (Coleoptera: Bruchidae)	240	6.58 (5.43–8.46)	180	>100
Sitophilus oryzae (Coleoptera: Rhynchophoridae)	240	8.16 (7.11–9.14)	180	>100
Plutella xylostella (Lepidoptera: Yponomeutidae)	240	0.98 (0.85–1.13)	240	>100
Myzus persicae (Homoptera: Aphididae)	240	7.41 (7.01–7.85)	240	>100
Blatella germanica (Blattaria: Blattelidae)	120	1.33 (1.15–1.52)	60	>100
Tetranychus urticae (Acari: Tetranychidae)	240	1.24 (1.11–1.38)	120	>100

TABLE 3. SUSCEPTIBILITY OF *Reticulitermes speratus* NYMPHS AND *Sitophilus oryzae* FEMALE ADULTS TO CRUDE OIL AND CARVACROL DETERMINED BY DIFFERENT APPLICATION METHODS

	LD_{50} (mg/filter paper)					
	R. speratus				*S. oryzae*	
Method[a]	*N*	Crude oil	*N*	Carvacrol	*N*	Carvacrol
A	150	1.9	150	0.5	100	10.9
B	150	>50	150	5.7	100	>30
C	150	1.3	150	0.3	100	9.7

[a] A: polyethylene cup contains filter treated with test materials, sea sand and lid; B: polyethylene cup contains filter paper treated with test materials, sea sand and no lid; C: polyethylene cup contains filter paper treated with test materials and lid.

the insecticidal mode of action of carvacrol may be attributable to fumigant action, although there is also significant contact toxicity based on our results.

Carvacrol could be of practical use as an insect-control agent in enclosed spaces such as storage bins, greenhouses, or buildings because of its fumigant action. An appropriate carrier would be needed to ensure slow release into the air for practical use, even in an enclosed area. Harding (1985) recommended a suitable liquid or solid carrier such as lacquer, sodium silicate, or paraffin. A combination of thujone oil and lacquer (12 : 88) used as a paint will keep rodents away from the treated area for between three and five years (Harding, 1985).

It has been well established that certain plant-derived extracts and phytochemicals may provide potential alternatives to currently used insect control agents (Jacobson and Crosby, 1971; Elliott, 1977; Hedin, 1982; Berenbaum, 1989). They have selectivity towards the natural enemies of pests, act in many ways on various types of pest complexes, and may be applied to the plant in the same way as other agricultural chemicals. Additionally, plant-derived materials are found to be highly effective against insecticide-resistant insect pests (Arnason et al., 1989b; Schmutterer, 1992; Ahn et al., 1997). The fumigant action of *T. dolabrata*-derived materials might be of importance in controlling stored-product insect pests. Although the methanol extract of *T. dolabrata* and carvacrol are known to inhibit human plasma cholinesterase (Katoh et al., 1986) and irritate human skin (Budavari et al., 1989), carvacrol has low toxicity (Budavari et al., 1989) and caused no mutagenicity when tested against four strains of *Salmonella typhimurium* Castel. and Chalm (Lee, 1993).

In conclusion, carvacrol may prove useful in the protection of some agricultural crops and stored products, provided that a carrier producing a slow-released effect can be selected or developed. An average of 4000 tons of sawdust per year is produced as a by-product of *T. dolabrata*, an important timber species in Japan (Kaburagi et al., 1969). Thus, there is an adequate source of the crude oil available.

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BEHAVIORAL (FEEDING) RESPONSES OF THE CRAYFISH, *Procambarus clarkii*, TO NATURAL DIETARY ITEMS AND COMMON COMPONENTS OF FORMULATED CRUSTACEAN FEEDS

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Abstract—We have examined the behavioral (feeding) response of *Procambarus clarkii* to natural dietary items (zooplankton, live fishes, dead fishes, and fish eggs) and common components of formulated feeds used in the aquaculture industry (soybean meal, fish meal, corn meal, alfalfa meal, and vitamin C). The feeding response by *P. clarkii* was determined using an ordinally ranked, whole-animal bioassay that included the following behaviors: (1) movement of the maxillipeds for longer than three seconds, (2) increased movement of the walking legs with dactyl "probing," (3) movement of walking legs to the mouth, and (4) orientation of the entire body towards the odor source. Feeding behavior was determined in response to intact items, bathwater containing aqueous leachates from intact items, water and methanol fractions of bathwater eluted through a C_{18} resin flash chromatography column, and size fractions of bathwater containing either molecules $\leq 10{,}000$ Da or molecules $> 10{,}000$ Da. All fractions tested were significantly stimulatory. Zooplankton was the most stimulatory of the natural dietary items tested. However, the C_{18} water fraction of the soybean meal bathwater before size fractionation (containing molecules both $< 10{,}000$ and $> 10{,}000$ Da) was the most stimulatory of the common feed components and more stimulatory than the zooplankton. Proximate analysis indicated that the compounds present in this fraction were ca. 51% soluble carbohydrate, 4% soluble protein, and 45% unknown (assumed to be insoluble carbohydrates, insoluble proteins, and ash). We hypothesize that the primary compounds in soybean meal responsible for eliciting a feeding response in *P. clarkii* are soluble carbohydrates and/or glycoproteins.

Key Words—Chemoreception, crustacean, crayfish, *Procambarus clarkii*, feeding, behavior, soybean.

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INTRODUCTION

In many aquatic ecosystems, crayfish are the dominant macrocrustacean (Momot et al., 1978). Their variable diets include nearly all taxonomic groups within the plant and animal kingdoms, as well as prokaryotes (Brown, 1995). Crayfish often consume organisms such as worms, aquatic insect larvae, small mollusks, amphibian tadpoles, fishes, fish eggs, small crustaceans, other crayfish, aquatic plants, detritus, and microorganisms associated with plants or detritus (King, 1883; Momot et al., 1978; Anderson and Sedell, 1979; Horns and Magnuson, 1981; Goddard, 1988; Savino and Miller, 1991; Brown et al., 1992).

Crayfish are also important economically. The crayfish aquaculture industry is well established, particularly in the southern United States. Crayfish are primarily raised in earthen ponds with a forage-based feeding system that relies on the growth of cultivated crops such as rice, and volunteer terrestrial and semiaquatic plants such as alligator weed and other detritus to provide food (Wiernicki, 1984; Goddard, 1988; Brunson, 1989; D'Abramo and Robinson, 1989; McClain et al., 1992; Huner, 1994). However, in forage-based feeding systems natural food stuffs may become limited seasonally. Growth and survival of crayfish increase when they are fed a supplemental diet formulated specifically for crustaceans (for review see D'Abramo and Robinson, 1989). Hence, formulated feeds could be used to intensify the crayfish aquaculture industry.

Despite the potential for the use of formulated feeds in crayfish culture, little is known about the dietary and nutritional requirements of crayfish. Similarly, little is known about crayfish feeding behavior, i.e., the ability to detect and locate food. Chemical stimuli have been shown to elicit feeding responses in crustaceans such as the marine lobsters *Homarus americanus* and *H. gammarus*, the marine shrimp *Palaemonetes pugio*, and the semi-terrestrial fiddler crab *Uca pugilator* (for review see Zimmer-Faust, 1989; Weissburg and Zimmer-Faust, 1991). Numerous studies have indicated that amino acids are the most stimulatory chemicals for eliciting feeding behavior in crustaceans considered to be largely carnivorous (Zimmer-Faust and Case, 1982; Zimmer-Faust et al., 1984a; Carr et al., 1989) and that sugars are the most stimulatory chemicals for eliciting feeding behavior in crustaceans considered to be largely herbivorous (Robertson et al., 1981; Trott and Robertson, 1984; Rittschof and Buswell, 1989). Peptides have also been well documented in stimulating important behavioral activities, including feeding, in aquatic animals (Rittschof, 1990). Moreover, mixture interactions, which can be either synergistic or suppressive, have been observed to alter the level of feeding responses shown by crustaceans in a number of chemoreception studies (for review see Carr and Derby, 1986a). Feeding behavior in response to synthetic solutions of prey extracts, composed only of amino acids, is less pronounced than in response to natural extracts (McLeese, 1970; Carr, 1978; Carr and Derby, 1986b). The reduced induction

of feeding behavior by incomplete synthetic solutions may be due to the absence of some signaling peptide, which only in conjunction with other compounds produces the maximal response (Zimmer-Faust et al., 1994). Carr and Derby (1986b) observed that two stimulatory chemicals presented in a single solution evoke a much stronger response than that predicted by additive effect calculations based on the individual stimulatory activity of the two chemicals.

In an effort to further our understanding of an organism that is important both ecologically and economically, we have examined the chemoreception capability of the Louisiana red swamp crayfish, *Procambarus clarkii*, in relation to feeding behavior by presenting natural dietary items and common formulated crustacean feed components. An ordinal progression of the behavioral (feeding) response by *P. clarkii* was developed as a whole-animal bioassay indicative of feeding by *P. clarkii*. Intact items and bathwaters containing aqueous leachates of intact items were tested to determine their effect in stimulating a behavioral feeding response. The most stimulatory bathwaters were further analyzed, and the proximate chemical characteristics of the compounds capable of stimulating a feeding response in *P. clarkii* were determined.

METHODS AND MATERIAL

Determination of Behavioral (Feeding) Responses in Procambarus clarkii. Adult *P. clarkii* of both sexes (N = 150, ca. 15 g wet weight) were obtained from Dr. Robert Romaire, Louisiana State University, and used in whole-animal bioassays. Crayfish were held in a 1000-liter (aged tap water) common tank (22–23°C) and fed daily a feed formulated specifically for crayfish (KS diet, UAB Research Foundation). On test days the crayfish were not fed at least 12 hr prior to experimentation. All experiments were performed with intermolt individuals. No individual was used more than once for the same test item.

Individual crayfish (N = 15 for each item or fraction tested) were exposed to test items in Plexiglas tanks (40 cm long × 25 cm wide × 14 cm deep) containing 4 liters of aged tap water (22–23°C). Screening mesh on the tank bottom provided traction for the crayfish (Figure 1). A 9-cm × 25-cm odor (stimulus) delivery area was separated from the rest of the tank by an opaque Plexiglas divider perforated by a rectangular array of ca. 30 evenly spaced 1-cm-diameter holes. The holes enabled water and particle movement into the area of the tank occupied by the crayfish as well as test item manipulation by the crayfish.

Crayfish were haphazardly removed from the common tank for testing. Each crayfish was placed in an individual Plexiglas tank and allowed to acclimate (minimum 15 min) until no movement was detected. Before the item to be tested was presented into the odor delivery area, the response of each crayfish

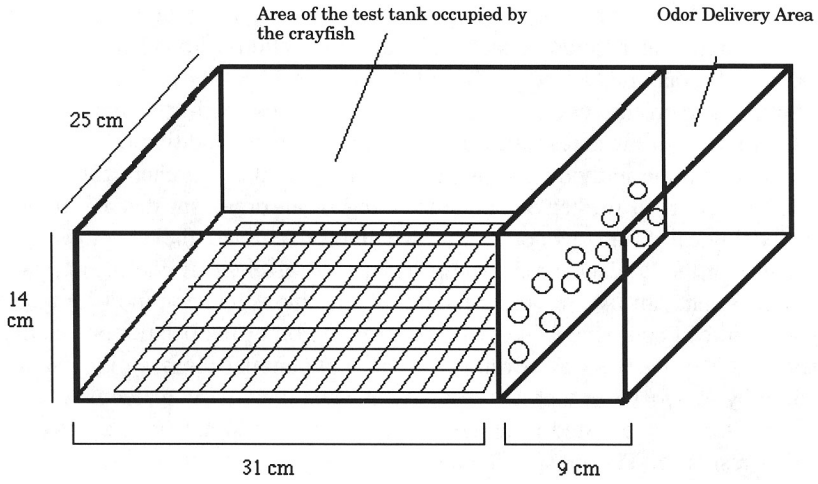

FIG. 1. Plexiglas test tank in which all experiments were performed. Intact items or bathwater leachates were placed into the odor delivery area, mixed, and allowed to diffuse through the porous partition and into the area of the test tank occupied by the crayfish.

to the delivery of aged tap water without additions for a 3-min interval was recorded.

After a test item was introduced into the tank via the odor delivery area, each crayfish was allowed 3 min to respond to the item presented. The behavioral (feeding) responses of each individual crayfish were ranked based on four ordinal levels of typical feeding behavior observed in preliminary tests in which crayfish were proffered the KS diet, which they readily consumed. We considered these feeding behaviors to be "typical" as they were identical to the behaviors performed when natural food items were presented to the crayfish. The behaviors used to determine the level of feeding response were: (1) movement of the maxillipeds for longer than 3 sec, (2) increased movement of the walking legs with dactyl "probing," (3) movement of walking legs to the mouth, and (4) orientation of the entire body towards the odor source. If the crayfish did not respond to a test item, its capability to respond was verified by presentation of the KS diet, which had been observed to elicit a level 4 maximal response. Crayfish that did not respond to the KS diet with a response level of 4 were not included in the data analysis. The Wilcoxon signed-rank test was used to determine if a significant difference existed between the responses of the crayfish to each test item and the control (water addition).

Feeding Response of Procambarus clarkii *to Intact Natural Dietary Items and Common Formulated Feed Components.* Four natural dietary items of cray-

fish, as determined from the literature, were tested. These included adult, flash-frozen brine shrimp (as a zooplankton model), freshly killed fishes (*Oreochromis niloticus*) with postmortem lacerations, live fishes (*O. niloticus*), and fish (*O. niloticus*) eggs. Five common formulated crustacean feed components were also tested, including soybean meal, fish meal (menhaden), corn meal, alfalfa meal, and vitamin C. Ten grams of each intact prey item or feed component was presented into the odor delivery area of a tank containing an individual crayfish, and the water was stirred with a glass rod for a few seconds to facilitate dispersal of the item. The opaque partition dividing the odor delivery area from the portion of the tank occupied by the crayfish had openings large enough to allow the item to be handled physically by the crayfish. The control consisted of adding aged tap water to the odor delivery area and stirring the water with a glass rod for a few seconds. The ordinal response for each item tested, including the control was recorded.

Feeding Response of Procambarus clarkii *to Bathwater Containing Aqueous Leachates from Intact Dietary Items and Formulated Feed Components.* Individual bathwaters were prepared by placing 200 g of each test item (except fish eggs due to insufficient amount of material) into 800 ml of aged tap water and allowing it to sit for 1 hr at 4°C. The solutions were then filtered (0.45 μm Millipore) to remove particulates. Twenty milliliters of bathwater was presented to a crayfish ($N = 15$ for each item tested) via the odor delivery area. The control was 20 ml of aged tap water. The ordinal response for each bathwater tested, including the control, was recorded.

Feeding Response of Procambarus clarkii *to Fractions of Stimulatory Bathwater Containing Aqueous Leachates.* The two most stimulatory bathwaters (soybean meal and brine shrimp leachates) as determined in the previous experiment were fractionated by reverse-phase (C_{18} resin), flash chromatography. Three hundred fifty milliliters of bathwater (prepared as previously stated) from each item was fractionated on a C_{18} column (binds more lipophilic compounds). A water fraction (ca. 350 ml) was first collected. The water fraction consisted of those compounds that did not bind to the C_{18} resins and hence were eluted through the column. A methanol fraction was then collected by rinsing the column with 2 volumes of 100% methanol. Methanol fractions were concentrated with a rotovap, dried under nitrogen, and then resolubilized in the same volume of aged tap water as the eluted water fractions (ca. 350 ml). Twenty milliliters of each fraction were presented to a crayfish ($N = 15$ for each fraction tested). The control was aged tap water. The ordinal response for each fraction tested, including the control, was recorded. The most stimulatory fraction was determined to be the C_{18} water fraction of the soybean meal bathwater. This fraction was used in all further analysis.

Size Characterization of Stimulatory Compounds. Ultrafiltration was performed on the most stimulatory fraction (C_{18} water fraction of the soybean meal

bathwater) using a pressure ultrafiltration vessel (Amicon model 8400) and a UM-10 (Amicon) membrane at 4°C under 60 psi of nitrogen. Three hundred fifty milliliters of the C_{18} water fraction were placed into the pressure vessel. When 100 ml of the original fraction remained as ultraretenate, 100 ml of aged tap water was added to the pressure vessel to further dilute the remaining ultraretenate and facilitate the removal of all molecules with a molecular weight equal to or less than ca. 10,000 Da. This produced two fractions, an ultrafiltrate containing molecules equal to or less than ca. 10,000 Da and an ultraretenate containing molecules greater than ca. 10,000 Da (although it is possible that some molecules less than 10,000 Da still remained in the ultraretentate). Twenty milliliters of each fraction was presented to a crayfish ($N = 15$ for each fraction tested). A recombined solution containing both size fractions was also tested. The control was aged tap water. The ordinal response for each size fraction tested, including the control, was recorded.

Dose Dependency of Stimulatory Compounds. Trials were performed to determine the magnitude of the crayfish feeding responses to a dilution series of the most stimulatory bathwater fraction. Three hundred fifty milliliters of the C_{18} water fraction of the soybean meal bathwater was frozen at −80°C and then lyophilized. The resulting light brown, crystalline-like powder was weighed and the concentration of the stimulatory powder was determined to be 41 mg/ml bathwater. A dilution series (1.0×, 0.1×, 0.01×, 0.001×, 0.0001×, and 0.00001× the original concentration) of the crystalline powder was resolubilized in aged tap water. Twenty milliliters of each solution was presented to a crayfish ($N = 15$ for each solution). Since the test tanks contained 4 liters of water, stimulus solutions could be maximally diluted 200 times. Consequently, a solution with a concentration of 41 mg/ml (1.0×) could reach a concentration as low as 0.21 mg/ml after complete diffusion of the 20 ml of test solution in 4 liters of tank water. Therefore, actual concentrations to which crayfish were exposed and responded to may likely have been less than the concentrations indicated in the results.

Proximate Biochemical Analysis of the Most Stimulatory Fraction. The percentage of soluble protein and carbohydrate in the lyophilized sample was determined with methods by Bradford (1976) and Dubois et al. (1956), respectively.

RESULTS

Feeding Response of Procambarus clarkii *to Intact Natural Dietary Items and Common Formulated Crustacean Feed Components. P. clarkii* showed a significant feeding response ($P \leq 0.001$) to all of the items tested (Table 1). Brine shrimp elicited the highest percentage of level 4 responses (80%; average

TABLE 1. BEHAVIORAL (FEEDING) RESPONSES OF *Procambarus clarkii* TO INTACT NATURAL DIETARY ITEMS AND COMMON COMPONENTS OF FORMULATED CRUSTACEAN FEEDS[a]

	Percentage showing response				Mean max. test response[b]	Mean control response	*N*
	1	2	3	4			
Feed Components							
Soybean meal	100	100	100	93	3.9	0	15
Fish meal	100	87	87	73	3.5	0	15
Corn meal	93	87	80	73	3.3	0	15
Alfalfa meal	100	93	87	67	3.5	0	15
Vitamin C	100	93	60	27	2.8	0	15
Dietary items							
Brine shrimp	100	93	93	80	3.7	0	15
Fresh kill fishes	100	100	79	71	3.5	0	14
Live fishes	100	100	80	53	3.3	0	15
Fish eggs	100	80	60	33	2.7	0	15

[a]Feeding responses are: (1) movement of the maxillipeds for >3 sec, (2) increased movement of the walking legs with dactyl probing, (3) movement of walking legs to the mouth, and (4) orientation of the entire body towards the odor source.
[b]The difference is significant between the average test responses of cryfish to all test items vs. the control. $P \leq 0.001$.

feeding response = 3.7) for the natural dietary items, while soybean meal elicited the highest percentage of level 4 responses (93%; average feeding response = 3.9) for the formulated feed components.

Feeding Response of Procambarus clarkii *to Bathwater Containing Aqueous Leachates from Intact Dietary Items and Formulated Feed Components. P. clarkii* showed a significant feeding response ($P \leq 0.01$) to all of the bathwaters tested (Table 2). Brine shrimp elicited the highest percentage of level 4 responses (13%; average feeding response = 2.9) for the bathwaters of the natural dietary items, and soybean meal elicited the highest percentage of level 4 responses (33%; average feeding response = 3.1) for the bathwaters of the formulated feed components. The two most stimulatory bathwaters, brine shrimp and soybean meal, were the only bathwaters used in further analysis.

Feeding Response of Procambarus clarkii *to Fractions of Stimulatory Bathwater Containing Aqueous Leachates.* When compared to the control, both the methanol and water fractions of the brine shrimp bathwater elicited a highly significant feeding response ($P = 0.0006$ and $P = 0.0005$, respectively) (Table 3) in *P. clarkii*. The response elicited by the methanol fraction was significantly lower than the response elicited by the water fraction ($P = 0.008$, Wilcoxon signed-rank test). Moreover, the C_{18} water fraction of the brine shrimp elicited a slightly higher percentage of level 4 responses (13%) than did the C_{18} methanol fraction of the brine shrimp (7%). The percentage of level 4 responses (13%) elicited by the water fraction of the brine shrimp was the same as the percentage of level 4 responses (13%) elicited by the complete brine shrimp bathwater before fractionation by C_{18} flash chromatography.

When compared to the control, both the methanol and water fractions of the soybean meal bathwater elicited a significant feeding response ($P = 0.01$ and $P = 0.0005$, respectively). The response elicited by the methanol fraction was significantly lower than the response elicited by the water fraction ($P = 0.0008$). The C_{18} water fraction of the soybean meal also elicited a higher percentage of level 4 responses (33%) than did the C_{18} methanol fraction of the soybean meal (7%). In addition, the percentage of level 4 responses (33%) elicited by the water fraction of the soybean meal was the same as the percentage of level 4 responses (33%) elicited by the complete soybean meal bathwater before fractionation by C_{18} flash chromatography.

Based on the percentage of level 4 responses elicited by each of the fractions, the C_{18} water fractions of both brine shrimp and soybean meal were more stimulatory than either of the C_{18} methanol fractions. The soybean meal C_{18} water fraction was more stimulatory than the brine shrimp C_{18} water fraction. The C_{18} water fraction of the soybean meal was the only fraction used in further analysis.

Size Characterization of Stimulatory Compounds. When compared to the control, both the ultrafiltrate (containing molecules $\leq 10{,}000$ Da) and ultra-

TABLE 2. BEHAVIORAL (FEEDING) RESPONSES OF *Procambarus clarkii* TO BATH WATER CONTAINING AQUEOUS LEACHATES FROM NATURAL DIETARY ITEMS AND COMMON COMPONENTS OF FORMULATED CRUSTACEAN FEEDS[a]

	Percentage showing response				Mean max. test response[b]	Mean control response	*N*
	1	2	3	4			
Feed components							
Soybean meal	100	93	80	33	3.1†	0	15
Fish meal	100	80	80	27	2.9†	0	15
Corn meal	100	93	80	20	2.9†	0	15
Alfalfa meal	87	60	20	07	1.7†	0	15
Vitamin C	100	93	20	07	2.4†	0	15
Dietary items							
Brine shrimp	100	100	80	13	2.9†	0	15
Fresh kill fishes	93	67	13	0	1.7†	0	15
Live fishes	87	60	07	0	1.5*	0	15
Fish eggs	nt	nt	nt	nt	nt	nt	nt

[a] Feeding responses are: (1) movement of the maxillipeds for >3 sec, (2) increased movement of the walking legs with dactyl probing, (3) movement of walking legs to the mouth, and (4) orientation of the entire body towards the odor source, nt = not tested.

[b] The difference is significant between the average test responses of crayfish to all test items vs. the control: $*P \leq 0.01$; $\dagger P \leq 0.001$.

TABLE 3. BEHAVIORAL (FEEDING) RESPONSES OF *Procambarus clarkii* TO WATER AND METHANOL FRACTIONS OF SOYBEAN MEAL BATH WATER ELUTED THROUGH A C_{18} FLASH CHROMATOGRAPHY COLUMN[a]

	Percentage showing response				Mean max. test response[b]	Mean control response	*N*
	1	2	3	4			
Soybean meal							
Water fraction	100	93	47	33	3.1	0	15
Methanol fraction	47	40	07	07	1.0	0	15
Brine shrimp							
Water fraction	100	93	60	13	2.7	0	15
Methanol fraction	100	87	27	07	2.2	0	15

[a]Feeding responses are: (1) movement of the maxillipeds for >3 sec, (2) increased movement of the walking legs with dactyl probing, (3) movement of walking legs to the mouth, and (4) orientation of the entire body towards the odor source.
[b]The difference is significant between the average test responses of crayfish to all test items vs. the control. $P < 0.001$.

retenate (containing molecules >10,000 Da) fractions, obtained from the C_{18} water fraction of soybean meal bathwater, elicited a significant feeding response ($P = 0.002$ and $P = 0.001$, respectively) (Table 4). There was no significant difference between the responses to the ultrafiltrate and the ultraretentate fractions. Both the ultrafiltrate and the ultraretentate size fractions tested individually were less active than the C_{18} water fraction tested before ultrafiltration, this difference, however, was not significant ($P = .06$ and $P = .35$). A recombined fraction containing both the ultrafiltrate and ultraretenate elicited an average feeding response and percentage of level 4 responses about equal to those elicited by the C_{18} water fraction of the soybean meal bathwater before ultrafiltration. The responses to this recombined fraction were also the same as those reported for the original soybean meal bathwater before C_{18} fractionation or ultrafiltration.

Dose-dependency of Stimulatory Compunds. Procambarus clarkii showed a dose-dependent response to the stimulatory compound(s) from the C_{18} water fraction of the soybean meal bathwater (Figure 2). Quantitatively, the C_{18} water fraction contained 41 mg of unidentified compound(s) per milliliter of the fraction prior to addition into the test tank. Responses of individuals to a dilution series of the original concentration indicated that individuals showed a significant response up to a 0.0001× dilution (4.1 μg/ml prior to addition into the test tank). The difference in the percentage of maximal response levels elicited by each concentration is shown in Figure 3.

Proximate Biochemical Analysis of the Most Stimulatory Fraction. Powder from a lyophilized sample of the C_{18} water fraction of the soybean meal bathwater contained 4.2% soluble protein and 50.9% soluble carbohydrate. Since most lipophilic compounds should have been partitioned into the less stimulatory methanol fraction during C_{18} flash chromatography, we suggest the remaining 44.9% is insoluble carbohydrates, insoluble proteins, and ash.

DISCUSSION

Several studies have examined the feeding responses of decapod crustaceans to various substances by using whole-animal bioassays (for review see Zimmer-Faust, 1989). Some of these studies have reported that various decapods exhibit stereotypical, sequential behaviors that lead to consumption of the stimulus (Hindley, 1975; Derby and Atema, 1981; Zimmer-Faust et al., 1984b; Harpaz et al., 1987; Zimmer-Faust, 1987). However, a response was usually reported as positive only when a complete sequence of events occurred, which might have included the following responses: increased antennular flicking, followed by food searching motions of the first walking legs, movement of periopods towards the mouth parts and movement of the individual to the stimulant release point. The behaviors outlined by Harpaz et al. (1987), and reported

TABLE 4. BEHAVIORAL (FEEDING) RESPONSES OF *Procambarus clarkii* TO ULTRAFILTRATION FRACTIONS OF A C_{18} WATER FRACTION OF SOYBEAN MEAL BATH WATER[a]

	Percentage showing response				Mean max. test response[b]	Mean control response	*N*
	1	2	3	4			
Less than *ca.* 10,000 mol. wt.	86	79	43	7	2.1*	0	14
Greater than *ca.* 10,000 mol. wt.	93	79	64	29	2.6†	0	14
Recombined fractions	100	87	73	33	3.0†	0	15

[a]Feeding responses are: (1) movement of the maxillipeds for >3 sec, (2) increased movement of the walking legs with dactyl probing, (3) movement of walking legs to the mouth, and (4) orientation of the entire body towards the odor source.

[b]The difference is significant between the average test responses of crayfish to all test items vs. the control: $*P \leq 0.01$; $\dagger P < 0.001$.

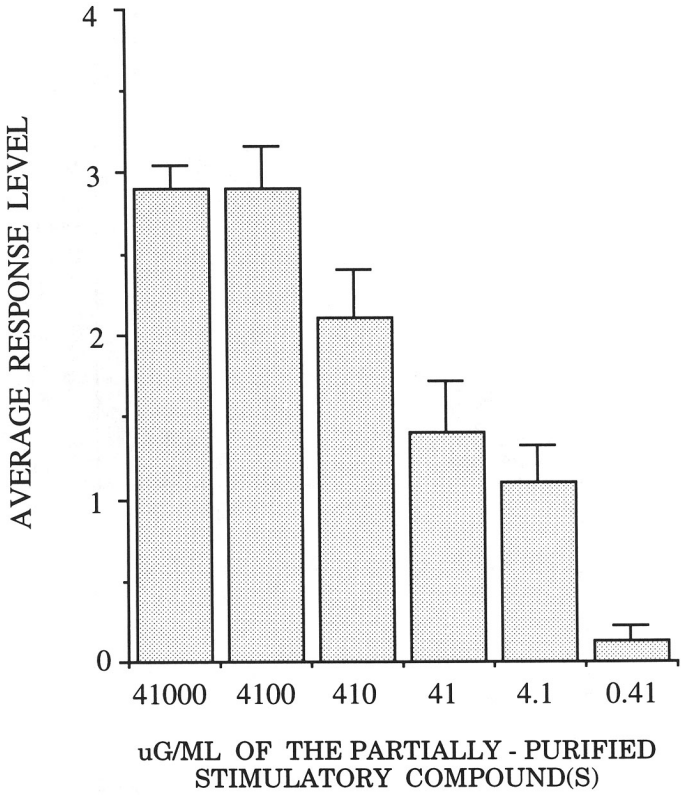

FIG. 2. Dose-dependent response by *P. clarkii* to a dilution series (1.0×, 0.1×, 0.01×, 0.001×, 0.0001×, and 0.00001×) of the C_{18} water fraction of the soybean meal bathwater. Standard error bars are included.

response sequences from other studies (Hindley, 1975; Derby and Atema, 1981; Zimmer-Faust et al., 1984b) parallel closely the bioassay used in this study. However, this study is the first report of a whole-animal bioassay based on a series of ordinally ranked behaviors, each recorded as a specific level of response, for the determination of a feeding response in the Louisiana red swamp crayfish, *Procambarus clarkii*, or any other decapod crustacean. Using this bioassay, the intensity of the feeding response of *P. clarkii* to comparable amounts of various natural dietary items and common components of formulated crustacean feeds was determined. This bioassay was developed from observations made during preliminary experimentation. When a stimulatory item such as the KS diet was presented to *P. clarkii*, the crayfish exhibited a predictable series of four sequen-

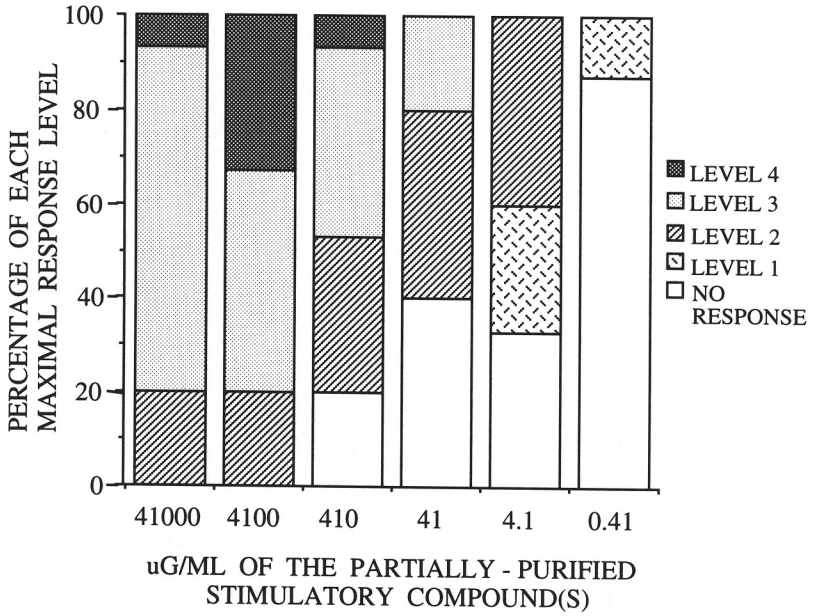

FIG. 3. Changes in the maximal response of the 15 individuals tested throughout a dilution series (1.0×, 0.1×, 0.01×, 0.001×, 0.0001×, and 0.00001×) of the C_{18} water fraction of the soybean meal bathwater.

tial responses. The occurrence of each response level was dependent on the occurrence of the level before it, i.e., a level 3 response occurred after level 1 and 2 responses, respectively.

Response level 1, movement of maxillipeds for longer than 3 sec, indicated chemical detection by the crayfish. A positive response was required to last longer than 3 sec, because it was observed that any slight mechanical disturbance of the water could result in short term (1- to 2-sec) maxilliped movement. However, the presence of stimulatory compounds resulted in sustained maxilliped movement for longer than 3 sec. Antennule flicking, a common crustacean indicator of chemical detection, was readily observed in *P. clarkii*. However, antennule flicking always leads to a level 1 response at the concentrations of stimuli tested in this study, and therefore was not used as a single response indicator. Response level 2, increased movement of the walking legs with dactyl probing, indicated a food searching response. This response allowed the crayfish to explore different parts of their immediate environment by sampling the substrate for food items. Response level 3, movement of the walking legs to the mouth, suggested an attempted consumption of food items, or alternatively, a

taste response. Apparently, after the crayfish has detected a threshold concentration, it begins these movements whether any solid particles are present or not. The item that the leg was holding was then ingested or rejected. If no item was being held by the leg, or if the item was not of a food origin, the crayfish quickly rejected it and resumed more active searching movements. Response level 4, orientation of the entire body towards the odor source, was the maximal response possible in this bioassay. Individuals displaying a level 4 response exhibited rheotactically directed chemotaxis, and orientation occurred only when the individual moved directly towards the odor source as opposed to exhibiting random search patterns leading to accidental discovery of the odor source.

In the presence of a strong feeding stimulus, such as soybean meal, once a level 1 response was exhibited, a level 4 response might be reached within seconds. In the presence of weaker feeding stimuli, the movement from one response level to the next might take several minutes. Although variations in the response times for both the onset of a feeding response and the time between the elicitation of higher response levels were not recorded, it would be interesting to consider these temporal variations of behavior in future studies.

The whole-animal bioassay used in this study recognizes and defines four distinct behaviors that are necessary for the successful location of food by *P. clarkii*. Moreover, this bioassay may have some advantageous qualities for use in the study of *P. clarkii* chemoreception. For example, the bioassay allows for some degree of discrimination between the levels of the feeding responses as compared to an all or none response determination. In addition, although the "stimulation" difference between levels is not easily quantified, each level indicates a higher intensity of feeding response and allows for qualitative comparisons between test items that may differ in their stimulatory capabilities. Qualitative comparisons may be important because, as this study demonstrates, items that are significantly stimulatory (compared to a control) do not always invoke the same intensity of response.

This study has shown that *P. clarkii* is capable of responding to a wide variety of potential food items. This is not surprising for an organism that is an omnivore and therefore should be able to detect a wide range of prey items. Adult brine shrimp (flash frozen) and soybean meal were the two items that elicited the most intense responses. Similar trends were observed using bathwaters that contained aqueous leachates. Although all other items produced some level of detectable feeding response, lower responses of *P. clarkii* to these other test items might be due to insufficient concentrations of soluble stimulatory compounds. However, since all items were tested at similar concentrations (weight to volume), there is little doubt that brine shrimp and soybean meal contain compounds with superior stimulatory capabilities for eliciting feeding responses in *P. clarkii*.

Intact particles and bathwater leachates of soybean meal generated a greater feeding response than the intact brine shrimp and its bathwater leachates. Evaluation of the C_{18} fractions (water and methanol) corroborates this trend. Aqueous leachates (water fractions) of both brine shrimp and soybean meal were more stimulatory than the methanol fraction of either test item. This would suggest that the compounds responsible for stimulating a feeding response in *P. clarkii* are water soluble. *P. clarkii* is an aquatic organism that must receive chemical cues from water-soluble (polar) compounds. Less polar compounds, such as those collected in the C_{18} methanol fractions, are unlikely to become soluble at concentrations high enough to be detected by organisms, resulting in a lower chance of detection, and therefore a reduced chance of being important chemoreceptively to the stimulation of a feeding response in an aquatic animal.

Ultrafiltration of the partially purified soybean meal leachate indicated that maximal stimulation of a feeding response by soybean meal is the result of several compounds of varying molecular weights. The response to each size fraction presented individually was less than the response to the complete fraction prior to ultrafiltration (the response to the complete fraction prior to ultrafiltration was greater than what would be predicted by additive effects). The response to the recombined fractions following ultrafiltration was similar to the response to the partially purified leachate before ultrafiltration. These data indicate a synergism may exist between small and large molecules, resulting in a maximimal feeding response shown by *P. clarkii*. Most studies in crustacean chemoreception have not tested molecules above 10,000 Da because all of the observable activity was attributed to molecules less than 10,000 Da (Carr and Gurin, 1975; Johnson and Ache, 1978; Robertson et al., 1981; Derby and Atema, 1982; Trott and Robertson, 1984). The results from this study indicate that molecules greater than 10,000 Da should be considered in future crustacean studies. A similar conclusion was reached by Zimmer-Faust et al. (1984b), who reported that macromolecules over 10,000 Da were tested and found to induce a stimulatory response in the lobster *Panulirus interruptus*. Because macromolecules are normally retained in larger chemical structures and are therefore not available for immediate release, it has been suggested that they could function as enduring indicators of food (Mackie, 1975), while small molecules (< 1000 Da) would be much less likely to function in this way due to their rapid rate of diffusion and selective assimilation by bacteria (Ogura, 1975). Zimmer-Faust et al. (1984a) suggest the longer lasting macromolecules may play an important role in facilitating food searching behavior among decapod crustaceans, especially for animals like the spiny lobster (and possibly crayfish) that alternate periods of foraging with inactivity.

The dose-dependent response of *P. clarkii* to the freeze-dried leachates of the C_{18} water fraction of soybean meal bathwater further validated the stimu-

latory nature of the compounds capable of eliciting a chemoreceptively mediated response. The minimum concentration to which *P. clarkii* showed a significant feeding response was between 4.1×10^{-3} mg/ml (before dilution in test tank) and 2.1×10^{-5} mg/ml (after dilution in test tank).

Based on the results of this study, the stimulatory compounds can be characterized as polar, varied in size (having molecular weights of both less than and greater than 10,000 Da), and expressing possible synergistic interactions. Proximate biochemical analysis of the freeze-dried leachate indicated that the stimulatory compounds are composed of 4% soluble protein, 52% soluble carbohydrate, and 44% unknown (hypothesized to be insoluble carbohydrates, protein, and ash). However, based on the proximate biochemical analysis and the crystalline, granular nature of the material recovered after freeze drying, we hypothesize that the primary compounds in soybean meal responsible for eliciting a feeding response in *P. clarkii* are soluble carbohydrates and/or soluble proteins with carbohydrate moieties (glycoproteins).

Carbohydrates have been shown to be stimulatory in other crayfish. Tierney and Atema (1988) used a whole-animal bioassay to determine the feeding response of two crayfish, *Orconectes virilis* and *O. rusticus*, to various amino acids and carbohydrates. They observed that carbohydrates were the most stimulatory compounds capable of eliciting a feeding behavior. Carbohydrates have been shown to elicit feeding responses in several other crustaceans as well (for review; see Tierney and Atema, 1988).

Soluble carbohydrates are a potentially important group of high-energy compounds for an omnivore such as *P. clarkii*. Chemical analysis of the dissolved organic material in freshwater lakes, a common habitat for crayfish, indicates that carbohydrates are quite abundant in the water column. Carbohydrate levels as high as 0.95–14.3 mg/ml have been found in the water column in various freshwater lakes (Ochiai and Handa, 1980a,b; Ochiai et al., 1980; Munster, 1984; Jorgensen, 1986). In fact, dissolved organic carbohydrates may comprise 5.8–42.3% of the total dissolved organic carbon in freshwater lentic systems (Alberts et al., 1988). One source for the carbohydrates present in lakes is decaying plant matter, which is a natural dietary item of crayfish and a commonly used forage material in commercial crayfish cultures. *P. clarkii* were capable of responding to low concentrations (between 4.1×10^{-3} mg/ml and 2.1×10^{-5} mg/ml) of stimulatory compounds. If the stimulatory compound from soybean meal is a carbohydrate, the levels to which *P. clarkii* responded are well below the reported, naturally occurring levels of total carbohydrates, indicating the specificity of the response by *P. clarkii* to the stimulatory compounds isolated in this study. It would also indicate that *P. clarkii* can show a feeding response to biologically relevant levels of the stimulatory compounds.

The presence of carbohydrate receptors in the branchial chambers of the

crayfish *Procambarus simulans* was indicated by Ashby and Larimer (1965), who found that bradycardia occurred following exposure to carbohydrates. Brown (1995) showed that crayfish have the enzymatic capability to digest and utilize carbohydrates. Carbohydrates are an important source of energy for any animal. They are functionally significant in glycogen storage, chitin synthesis, and the formation of steroids and fatty acids in crustaceans (Ali, 1993). Other investigators have shown that crayfish display a preference for plant feedstuffs, that contain large amounts of carbohydrates, as compared to animal feedstuffs that contain less carbohydrates and larger amounts of amino acids (Chidester, 1908; Covich, 1977; Brown et al., 1989). Nevertheless, other types of compounds are important in the diets of crayfish and may be involved in eliciting feeding responses. In fact, studies have shown that animals grow better when fed a diet containing both plant and animal materials (Kinne, 1959; Klekowski and Fischer, 1975). However, numerous studies on the dietary habits of a variety of crayfish leave little doubt that a large percentage of a crayfish's diet is composed of plant material and therefore carbohydrates (for review see Momot et al., 1978).

Most studies have examined proximate macronutrients as response stimuli in crustaceans. We found that the micronutrient vitamin C elicited a positive response in *P. clarkii*. In this study, crayfish demonstrated an immediate response to the addition of vitamin C. However, crayfish retreated from a concentrated vitamin C plume, presumably as a response to a low pH gradient caused by solubilization of the organic acid. Vitamin C is an important micronutrient in crustaceans (Lightner, 1993), and further studies are needed to determine the response and utilization of vitamin C as well as other micronutrients in crayfish.

Crayfish are a prominent species in the aquaculture industry throughout the world. Recent studies suggest that the use of formulated or supplemental feeds could increase the economic potential of the crayfish aquaculture industry. Understanding the chemoreceptive capabilities of crayfish could greatly facilitate the development of effective feeds for use in intensive culture systems or effective baits for existing methods of culture. The industry must understand which sensory cues attract crayfish to food and stimulate consumption. The formulation of a nutritionally balanced feed for crayfish will be of little value if the crayfish cannot effectively locate and willingly consume the feed. In semiintensive and intensive cultures, prompt discovery and consumption of the proffered feed will limit the risk of nutrient leaching of the feed and water quality deterioration due to uneaten feed accumulation. Stimulation of feeding will enhance growth and survival and ultimately increase the economic success of the culture.

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OLFACTOMETER-ASSESSED RESPONSES OF APHID *Rhopalosiphum padi* TO WHEAT AND OAT VOLATILES

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Abstract—Volatiles from wheat and oat seedlings elicited attraction in apterae and alatae *Rhopalosiphum padi*. Cereal volatiles were identified by GC-MS and olfactometric tests were performed with each compound. Attraction of aphids was elicited by (*E*)-2-hexenyl acetate, (*Z*)-3-hexenol, (*E*)-2-hexenyl acetate, (*E*)-2-hexenol, (*Z*)-2-hexenol, *n*-heptanal, *n*-octanal, *n*-nonanal, *n*-decanal, benzaldehyde, and linalool. The difference between the sensory capacity of alatae and apterae is discussed in relation to migrations between hosts during their life cycle.

Key Words—Odors, semiochemicals, green leaf volatiles, cereals, Hemiptera, Aphididae.

INTRODUCTION

Several studies have demonstrated a role for olfactory cues in host-plant selection by aphids prior to landing. Thus, attraction by host-plant odors was shown in *Aphis fabae* (Alikhan, 1960; Nottingham *et al.*, 1991), *A. gossypii* (Pospisil, 1972), and *Brevicoryne brassicae* (Pettersson, 1973) and *Cryptomyzus korschelti* showed positive upwind anemotaxis mediated by host-plant odors (Visser and Taanman, 1987). The chemicals responsible for the effects reported were not identified.

The bird-cherry-oat aphid, *Rhopalosiphum padi* (L.) (Hemiptera, Aphididae), is a holocyclic host-alternating species in temperate climates: bird-cherry, *Prunus padus* L. (Rosaceae), is the winter or primary host and a wide range of cereals and grasses (Poaceae = Gramineae) are its summer or secondary hosts.

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Odors play a role in this host alternating behavior. Thus, autumn migrants from the secondary host are attracted by odors from the winter host (Pettersson, 1993), the aggregation of *R. padi* colonies on *P. padus* is disrupted during the spring by methyl salicylate produced by the host (Pettersson *et al*, 1994), and parthenogenetic generations on the secondary host are attracted by host-plant odors (Quiroz *et al.*, 1997).

This report enquires into the composition of volatiles emitted by two summer hosts of *R. padi*, wheat and oat, and the behavioral responses they elicit in alatae and apterae *R. padi* in an olfactometer.

METHODS AND MATERIALS

Aphids. Aphids to start the cultures were collected in grass fields near the Laboratorio de Química Ecológica in Santiago, Chile. Cultures were kept on oat (*Avena sativa* L. cv. Nahuén) in a growth room at 18–22°C and light regime of 18L:6D. Winged forms were produced by wilting the oat plants by dehydration. In order to get individuals in an homogenous "searching behavioral mood," both alate and apterous morphs of similar size were collected from the cage walls and removed from the culture one hour before the experiment.

Plant Materials. Wheat (*Triticum aestivum* L. cv. Ciko) and oat seedlings were grown in a growth room at 18–22°C, light regime of 12H:12D, light intensity of 200 μmol photons/cm^{-2}/min and 45–65% relative humidity. Seedlings at growth stage 12 (Zadoks et al., 1974) were used for air entrainment experiments.

Chemicals. Samples used as stimulus in the olfactometer were purchased from Aldrich Chem. Co. and diluted in hexane before use.

Olfactometry. Behavioral studies were performed in an olfactometer as described by Pettersson (1970). One aphid was enclosed in an observation quadratic arena permeated by air (250 ml/min) coming from each of its four stretched-out corners and drawn out through a hole above its center. The treatment chemical was placed inside a test tube connected to the end of one of the arms; tubes with the control odors were connected to the other three arms. The pot with treatment seedlings was placed inside a bell jar connected to one of the olfactometer arms, and an empty control bell jar was connected to the other three arms. The carrier air was purified by passage through a device containing activated charcoal. The observation arena was divided into four arm zones and one indifferent zone in the center. The time the aphid spent in each arm was recorded during 15 min with the olfactometer being rotated every minute. Each experiment was replicated 10 times and results were analyzed using nonparametric statistics (Wilcoxon one-tailed rank-sum test for two groups), the total time spent in the treatment arm being compared with the mean time spent in the control

arms. An index was defined (equal to the ratio between time spent by the test aphid in the stimulus arm over time spent in the control arm) that reflected the behavioral effect of the stimulus, an index higher than 1 indicating attractiveness and an index lower than 1 indicating repellence.

Entrainment of Volatiles. Air was dried and purified by passage through activated 5 molecular sieves and charcoal, and drawn for 48 hr at 1 liter/min through two bell jars containing the odor sources (treatment and control). The treatment consisted of an intact plant in a plastic pot with soil and the control only a pot with soil. Volatiles were absorbed onto Porapak Q inside containers placed at the outlets of each bell jar and were desorbed by elution with freshly distilled ethyl ether (Blight, 1990). The resulting extract was concentrated under a stream of nitrogen and stored in seal ampoules at −20°C prior to analysis. Extracts or pure compounds (10 ng) diluted in hexane were applied onto pieces of Whatman No. 1 filter paper (2 cm^2), which were then placed inside the test tubes to be connected to the olfactometer arms.

Coupled Gas Chromatography—Mass Spectrometry. Two capillary GLC columns were used at different times: 25 m × 0.25 mm ID CBP20 and 25 m × 0.20 mm ID HP-1. The columns were directly coupled to a mass detector and an integrated data system (GC model HP-5890, MD model HP-5972). Ionization was electron impact at 70 eV and 280°C. The GC oven was maintained at 35°C for 5 min and then programmed to increase to 5°C/min to 200°C.

Each identified compound was quantified by interpolation from calibration curves obtained using four different dilutions (1, 25, 50, and 100 ng/μl) and docosane as internal standard. Quantitations were repeated three times on three different plant volatile extracts (standard errors below 10%).

Volatility of Pure Compounds in Olfactometer. Two series of samples of pure compounds (*ca.* 1 and 10 mg) were studied. Samples were applied onto pieces of Whatman No. 1 filter paper (2 cm^2) which were then placed inside test tubes to be connected to one of the arms of the olfactometer. Air was allowed to flow at 250 ml/min for 15 min. The filter paper was then removed and weighed. Three independent determinations were performed for each compound in each series. Average percentage vaporization did not differ between the series for every compound tested (Student's test). The values reported in Table 2 below correspond to those obtained using 1 mg of each compound.

RESULTS

The volatiles released from a group of 20 intact wheat seedlings and 20 intact oat seedlings elicited attraction in both alatae and apterae of *R. padi* in the olfactometer (Table 1, entries 1 and 5). Air entrainments of wheat and oat seedlings produced extracts whose constituents were identified by gas chroma-

TABLE 1. RESPONSE OF *Rhopalosiphum padi* APTERAE AND ALATAE TOWARDS OAT AND WHEAT VOLATILES IN OLFACTOMETER TESTS

	Apterae			Alatae		
Stimulus applied	Mean time spent in each arm (min)	Ratio stim./control	*P*	Mean time spent in each arm (min)	Ratio stim./control	*P*
20 Oat seedlings	4.71 ± 0.76	2.03	0.011	4.55 ± 0.86	2.26	0.0081
Blank	2.31 ± 0.83			2.01 ± 0.97		
Identified volatiles combined in the same ratio as released by oat (6.7 ng)[a]	3.56 ± 0.48	1.47	0.037	3.97 ± 0.69	1.31	0.045
Hexane	2.41 ± 0.63			3.02 ± 0.17		
Volatiles attractive to apterae combined in the same ratio as released by oat (4.5 ng)[a]	4.23 ± 0.54	1.20	0.047			
Hexane	3.53 ± 0.14					
Volatiles attractive to alatae combined in the same ratio as released by oat (1.5 ng)[a]				3.90 ± 0.44	1.31	0.045
Hexane				2.98 ± 0.42		

20 Wheat seedlings	4.30 ± 0.91	1.72	0.007	3.91 ± 0.81	1.82	0.049
Blank	2.50 ± 0.75			2.15 ± 0.85		
Identified volatiles combined in the same ratio as released by wheat (3.4 ng)[a]	3.95 ± 0.38	1.24	0.045	3.65 ± 0.41	1.20	0.045
Hexane	3.18 ± 0.21			3.04 ± 0.16		
Volatiles attractive to apterae combined in the same ratio as released by wheat (2.6 ng)[a]	3.83 ± 0.19	1.22	0.045			
Hexane	3.15 ± 0.51					
Volatiles attractive to alatae combined in the same ratio as released by wheat (0.6 ng)[a]				3.49 ± 0.86	1.16	0.151
Hexane				3.02 ± 0.26		

[a] Amount of volatiles released during 15 min from 20 seedlings of wheat or oat.

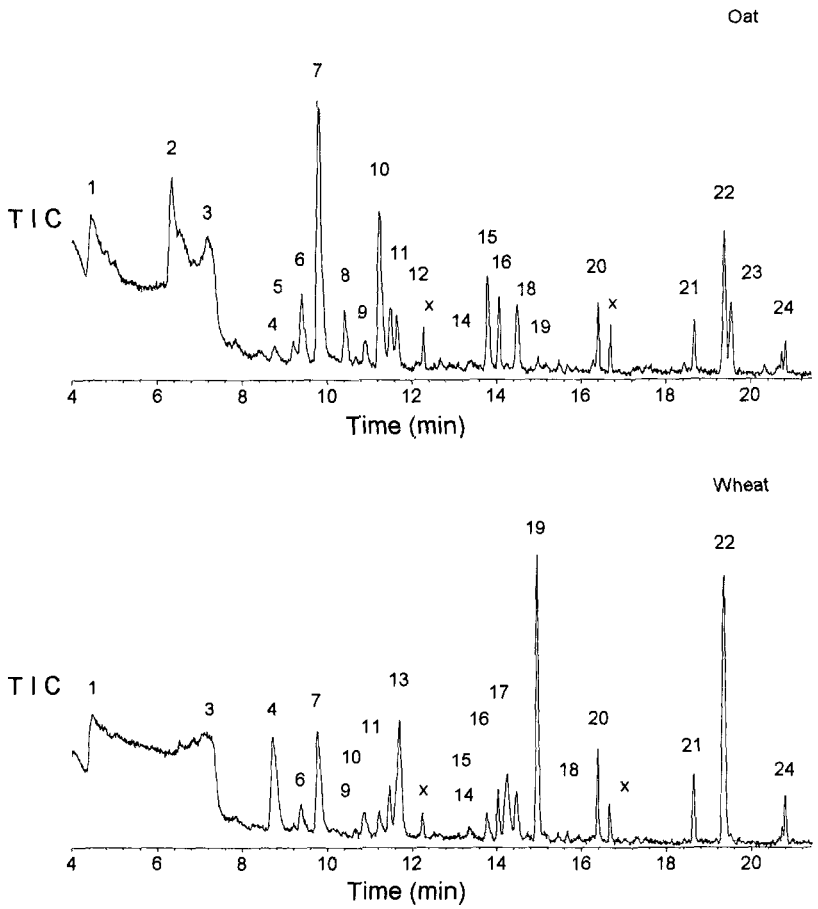

FIG. 1. Gas chromatographic analysis of Porapak-trapped volatiles from 20 intact wheat or oat seedlings using a 25-m × 0.25-mm ID CBP20 capillary column. Conditions are described in the Methods and Materials section. Peaks are identified in Table 1. *X* = compounds bleeding from the column.

tography coupled to mass spectrometry (Figure 1 and Table 2). Compounds were considered positively identified if peak enhancement occurred by coinjections with standard compounds, and their mass spectra and GLC Kovats indices were consistent with those from authentic samples analyzed under the same conditions. The average total release rates of volatiles were 330 and 641 ng/day/20 seedlings, for wheat and oat, respectively. Olfactory responses of apterae and alatae towards each of the volatile chemicals identified in the extract were

assessed in an olfactometer (Table 2). Volatile compounds that elicited attraction in apterae were (*Z*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, (*E*)-2-hexenol, (*Z*)-2-hexenol, *n*-heptanal, *n*-octanal, *n*-nonanal, *n*-decanal, benzaldehyde, and linalool, while volatile compounds which elicited attraction in alatae were (*E*)-2-hexenyl acetate, (*Z*)-3-hexenol, (*E*)-2-hexenol, and benzaldehyde. While most green leaf volatiles (GLV) and aldehydes were active, alkenes were inactive. All compounds showed volatilities above 40% in the olfactometer. Mixtures were produced in order to emulate natural host odors. In them, either all identified compounds or those active in the olfactometer were mixed in the average proportion found in the air entrainments from cereal seedlings. An amount of each mixture equivalent to that released by 20 wheat seedlings or 20 oat seedlings over a 15-min period elicited attraction in the olfactometer (Table 1, entries 2 and 6). Mixtures of volatiles individually eliciting attraction in alatae or apterae combined in the same ratio as released by the respective host plant elicited, with one exception (Table 1, entry 8), attraction in olfactometer tests (Table 1, entries, 3, 4 and 7).

DISCUSSION

The compounds identified belonged to five different categories: (1) green leaf volatiles, C-6 compounds arising from enzymic transformations of linolenic and linoleic acids (Hatanaka, 1993); (2) long-chain alkanes commonly present in plant epicuticular waxes; (3) common terpenoid plant constituents, such as linalool and camphor, (4) benzaldehydes, also common plant constituents, and (5) indene and naphthalene, two common environmental pollutants (Manahan, 1994). The present composition of cereal volatiles differs from those given in earlier reports for oat (Buttery et al., 1982) and wheat volatiles (Hamilton-Kemp and Andersen, 1984; Buttery et al., 1985). Since severed plants were used in the above investigations, some of the compounds reported may correspond to enzymic breakdown products of plant constituents (Schwimmer, 1981). The total amount of volatiles collected in the present work is on the order of 330 and 640 ng/day from ca. 10 g of fresh wheat and oat material respectively (Table 1). These amounts are comparable to those reported previously in the case of wheat (100–500 ng/day from 10 g of wheat leaves) (Buttery et al., 1985), but smaller than in the case of oat (ca. 2000 ng/day from 10 g of oat leaves) (Buttery et al., 1982). This latter differences may be ascribed to the age of the plants used; lower volatile production is expected from the younger plants used in the present work.

Most of the compounds identified were inactive in the olfactometer. Since the lack of activity of a given compound may be ascribed to a lack of volatility, the rate of vaporization of each compound was determined under the olfac-

TABLE 2. VOLATILES RELEASED FROM *Triticum aestivum* CV. CIKO AND *Avena sativa* CV. NAHUÉN AND THEIR EFFECT IN AN OLFACTOMETER TOWARDS *Rhopalosiphum padi* APTERAE AND ALATAE

Peak No.[a]	Compound	GLC Kováts index		Release rate (ng/day/20 seedlings)		Vaporization percentage in 15 min in the olfactometer	Olfactometer response (stimulus/control)[b]		Ratio of active volatiles released (oat/wheat)[c]
		CBP20	HP-1	Wheat	Oat		Alatae	Apterae	
GLVs									
1	*n*-Hexanal	1074	773	60.1	166.5	97	ns	1.3***	2.8
3	(*E*)-2-Hexenal	1214	809	32.1	75.0	97	ns	ns	
6	(*Z*)-3-Hexenyl ac.	1305	988	7.3	23.1	99	ns	2.0*	3.2
7	(*E*)-2-Hexenyl ac.	1323	995	24.0	81.6	83	2.3***	2.5*	3.4
8	(*E*)-3-Hexen-1-ol	1351	825		26.6	96	ns	ns	
9	(*Z*)-3-Hexen-1-ol	1371	831	6.4	11.1	98	1.8**	ns	1.7
11	(*E*)-2-Hexen-1-ol	1393	850	14.3	25.0	71	1.8**	1.9*	1.7
13	(*Z*)-2-Hexen-1-ol	1401	855	38.7		88	ns	1.7*	
Other components									
2	*n*-Heptanal	1172	902		40.3	95	ns	1.7***	
4	*n*-Octanal	1280	982	34.6	3.0	99	ns	1.5*	0.09

5	Tridecane	1301	1302		6.4	85	ns	ns	
10	*n*-Nonanal	1382	1081	5.3	44.6	60	ns	1.7*	8.4
12	Tetradecane	1402	1400		21.2	70	ns	ns	
15	*n*-Decanal	1489	1181	4.5	17.9	68	ns	1.4*	3.9
16	Pentadecane	1502	1501	7.3	22.1	60	ns	ns	
17	Camphor	1507	1121	9.4		81	ns	ns	
18	Benzaldehyde	1519	940	13.4	21.9	96	1.5*	1.3***	1.6
19	Linalool	1536	1083	43.4	4.0	74	ns	1.6***	0.09
20	Hexadecane	1605	1601	11.9	16.9	50	ns	ns	
21	Heptadecane	1702	1700	8.9	16.0	48	ns	ns	
23	4-Ethybenzaldehyde	1739	1147		9.6		ns	ns	
24	Octadecane	1801	1805	5.6	8.2	50	ns	ns	
Contaminants									
14	Indene	1472	1025	5.1	2.7	94	ns	ns	
22	Naphthalene	1731	1159	52.1	36.7	62	ns	ns	

[a] Peak numbers as in Figure 1.
[b] $P < 0.05$** $P < 0.01$*** $P < 0.001$ *ns* = non significant differences.
[c] These values were calculated from data in columns 5 and 6.

tometer conditions. All values were above 40% (Table 1), indicating that all compounds were sufficiently volatile to be present in the airstream of the olfactometer.

The activities in the olfactometer of artificial mixtures of volatile compounds (complete set of identified compounds or mixtures of compounds eliciting attraction in alatae and apterae) in the same quantities and proportions as released by oat and/or wheat seedlings were qualitatively the same as the plant headspace extracts (Table 1). However, the attraction elicited by the headspace extracts tended to be higher than the artificial mixtures (with the possible exception of Table 1, entry 8, which did not elicit a significant response). This suggests that the natural sources of volatiles (wheat and oat seedlings) emit other volatile(s) in quantities that were not detected by the method used and that either synergize the effect of other compounds present or elicit by themselves an attraction response.

Alatae and apterae showed different sensitivities toward volatiles from their host plants (Table 2). Electroantennographic studies on other aphid species have shown that different morphs may differ in their capacity to detect volatile semiochemicals. For example, while the antennal receptor system of alatae and apterae of *Sitobion avenae* (Fabr.) responded to alcohols containing six to seven carbon atoms, green leaf volatiles, and benzaldehyde, higher electroantennnographic responses were found for alatae (Yan and Visser, 1982), and in the aphid *Nasonovia ribis-nigris* different electroantennographic activities between alatae and apterae were found when the stimulus was hexanol or (*Z*)-3-hexen-1-ol (van Giessen et al., 1994).

The fact that attraction in *R. padi* alatae was elicited by four compounds and by 11 compounds in apterae suggests a basic difference in olfactory responses of the two morphs. We suggest that this olfactory specialization is in part related with the type of migration performed by each morph. Following take-off from a plant, flying alatae would need to be able to discriminate between a greater range of plant species than apterae, which tend to migrate locally within a reduced range of hosts (Wiktelius, 1989). Hence, alatae would improve their host-finding efficiency either by being tuned to specific volatile cues or, alternatively, to a set of common volatile cues in a given proportion. The results in Table 1 support this later alternative. Thus, while the ratios of "alatae-active" compounds in oat and wheat vary within a rather narrow range (between 1.5 and 3.4), the range of variation of ratios for "apterae-active" compounds is considerably larger [from 0.09 to 0.84 or more, as in the case of (*Z*)-2-hexen-1-ol and *n*-heptanal which were not even found in wheat extracts].

The results presented point to the involvement of olfactory cues in the early stages of host location by *R. padi*. The final choice of host will depend on the specificity of internal plant constituents assessed during probing (Niemeyer, 1990; Tjallingii, 1995).

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ELUCIDATION OF DIMETHYLALKYLPYRAZINES FROM THE ANT *Streblognathus aethiopicus* BY GC-FTIR

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Abstract—The presumed mandibular gland secretions from the workers and gamergates of two colonies of *Streblognathus aethiopicus* have been found to contain a homologous series of 3,5-dimethyl-2-alkylpyrazines (3,5-dimethyl-2-butylpyrazine, **1**; 3,5-dimethyl-2-pentylpyrazine, **2**; and 3,5-dimethyl-2-hexylpyrazine, **3**). The structures of these compounds were determined from their mass spectra along with a comparison of their GC-FTIR spectra with those of all the isomers of a lower homolog. This is the first time that this combination of techniques has been applied to the study of insect pyrazines. The relative amounts and proportions of these compounds were different for gamergates and workers, with the latter containing 2–10 times more total pyrazines and having a higher proportion of **2** and **3**. The possible significance of this finding is discussed.

Key Words—Pyrazines, GC-FTIR, *Streblognathus*, gamergate.

INTRODUCTION

The ant subfamily Ponerinae is distinctive in containing a small complex of genera in which the queen has been lost and her reproductive functions assumed by a single mated worker (Peeters, 1990). Monogynous queenless ponerines,

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characterized by the presence of a single gamergate or mated worker, have been detected in five genera, and there is strong evidence that these inseminated workers exert reproductive control over their nonmated sisters (Peeters, 1990; Ware, et al., 1990). Although gamergates may be morphologically indistinguishable from their nestmates, it is clear that major physiological differences distinguish them from the rest of the workers in the colony.

Recently, chemical analyses have revealed some exocrinological differences in the mandibular secretions of the workers and gamergates in a South American ponerine in the genus *Dinoponera* (Oldham, et al., 1994). In these ants, the mandibular secretion of the gamergate is 98% a single dimethylalkylpyrazine, while that of the workers, depending on age, may contain nearly equal amounts of up to four other pyrazines. Additionally, the mandibular gland secretions of gamergates of this *Dinoponera* species contain at least 50 times less detectable volatiles than those of the unmated workers.

Monogamergatous reproduction has also been demonstrated in the primitive ponerine *Streblognathus aethiopicus* (Ware et al., 1990), a genus in the same complex as *Dinoponera* (Carpenter, 1930) and data are available that compare the behavioral activities and reproductive functions for mated and unmated workers of this species. Although ovarian development and ovipositional behavior are manifested only in gamergates, the physiological bases for these differences are unknown for this species and other gamergatous ponerines (Ware et al., 1990). Indeed, the physiological and biochemical distinctions between mated and unmated workers in any gamergatous species are unknown.

Dimethylalkylpyrazines are typical components of the mandibular gland secretions of a variety of ant species (Blum, 1974; Wheeler and Blum, 1973; Longhurst et al., 1978). While the nature of the alkyl group in these compounds is often evident from their mass spectra (Brophy and Cavill, 1980), the substitution pattern on the aromatic ring is usually determined by comparison of their gas chromatographic retention times with those of authentic samples. This has always been the case in the assignment of the ring geometry of dimethylalkylpyrazines isolated from insects. (Duffield et al. 1981; Longhurst et al. 1978; Wheeler and Blum, 1973). It has been demonstrated that the elution order of these compounds on polar and nonpolar gas chromatography columns is 2,5-dimethyl-3-alkylpyrazines, followed by 3,5-dimethyl-2-alkylpyrazines, and finally the 2,3-dimethyl-5-alkylpyrazines (Mihara and Masuda, 1987). While these compounds are frequently found in social insects, in many cases it is not practical to have samples of all three isomers available for gas chromatographic comparison. In the present report, we describe a comparative investigation of the mandibular products of gamergates and unmated workers of *Streblognathus aethiopicus*. In essence, we wished to determine whether insemination of a worker (=gamergate) would result in either quantitative or qualitative changes in exocrine products vis-à-vis the unmated worker. In the case of *S. aethiopicus*,

analysis of the mandibular secretions has provided an opportunity to investigate the GC-FTIR of the dimethylalkylpyrazine isomers as a method for determining their geometry directly.

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic analyses were performed using a Shimadzu GC-9a equipped with a 30-m × 0.5-mm-ID open DB-17 column with a 1-μm film thickness. Mass spectra were obtained with an LKB-2091 GC-MS or a Finnigan ion-trap (model 800). Both instruments were equipped with 25-m × 0.32-mm-ID HP-5 fused silica columns. FTIR spectra were obtained using a Hewlett-Packard model 5965B detector (narrow band, 4000–750 cm^{-1}, resolution 8 cm^{-1}) interfaced with a Hewlett-Packard 5890 gas chromatograph.

Ants. Two complete colonies of *Streblognathus aethiopicus* were collected at Burnt Kraal near Grahamstown, South Africa. One colony contained 43 unmated workers and the other 48. A single gamergate was collected from each colony. Egg-laying by these individuals and their attractiveness to nonmated workers clearly identified them as true reproductives. Each ant was decapitated, and the individual heads placed in separate vials of methylene chloride. A total of 93 individual ants was examined. For analysis, the volume of the solvent was reduced to ca. 0.1 ml with a gentle stream of nitrogen.

RESULTS AND DISCUSSION

Mass spectral analysis of the methylene chloride extracts revealed the presence of three homologous *n*-alkyldimethylpyrazines. These were readily identified as a dimethyl-*n*-butylpyrazine (**1**), a dimethyl-*n*-pentylpyrazine (**2**), and a dimethyl-*n*-hexylpyrazine (**3**) from their mass spectra (Brophy and Cavill, 1980; Chiu et al., 1990). In addition, a very small amount (<2%) of a dimethyl-*n*-propylpyrazine was detected in most cases.

Since the mass spectra of alkyldimethylpyrazines reveal little if anything of their geometries, the GC-FTIR of the pyrazines from *S. aethiopicus*, dimethylalkylpyrazines **1**, **2**, and **3**, were obtained (Figure 1). It is readily evident from the FTIR spectra that these pyrazines belong to a homologous series and that they differ only in the length of the side chain (butyl, pentyl, and hexyl, respectively, from GC-MS). The frequencies of absorptions (cm^{-1}) are the same, and there is an increase of intensity (absorbance) for the C–H stretching vibrations (2800–3050 cm^{-1}) with respect to the fingerprint region (1000–1500 cm^{-1}) with increasing length of the side chain from butyl to hexyl.

Although the three isomers each of **1**, **2**, and **3** were not available for

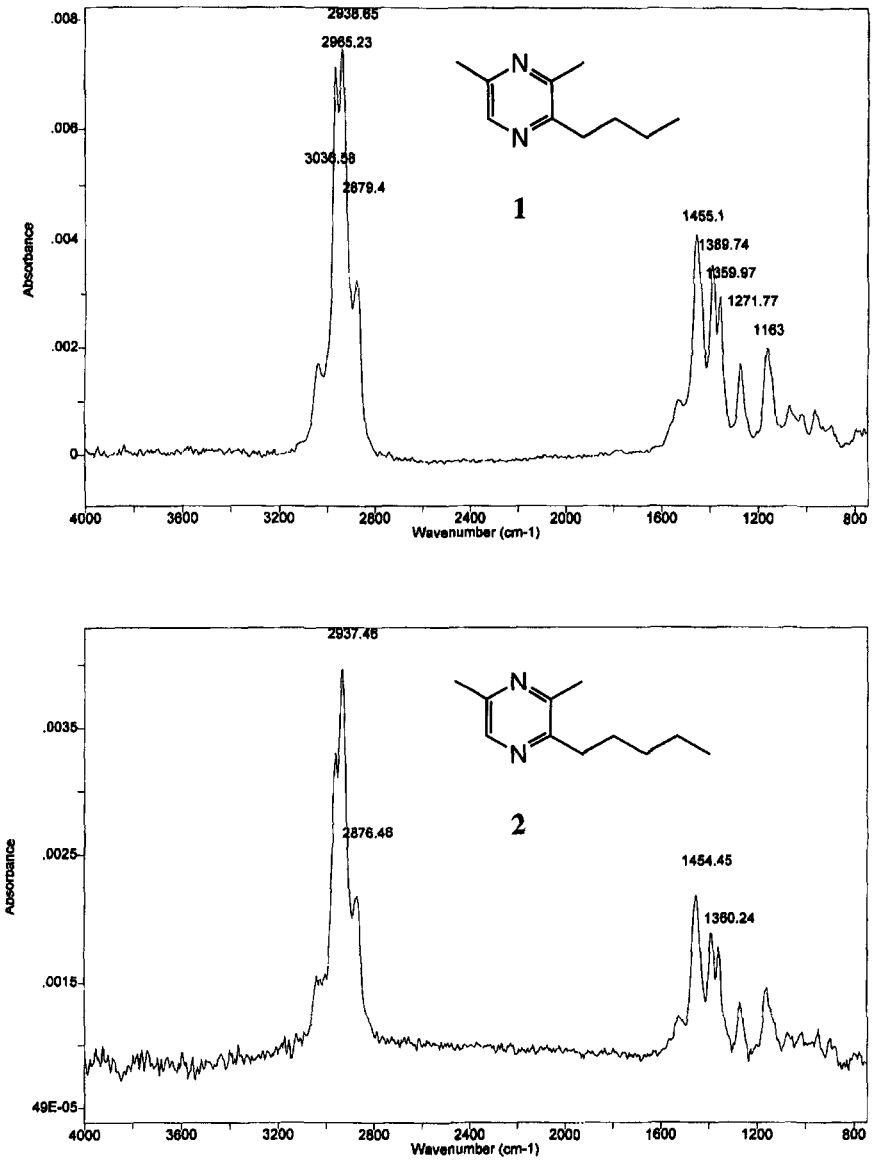

FIG. 1. GC-FTIR spectra of pyrazines **1**, **2**, and **3** from *Streblognathus aethiopicus*.

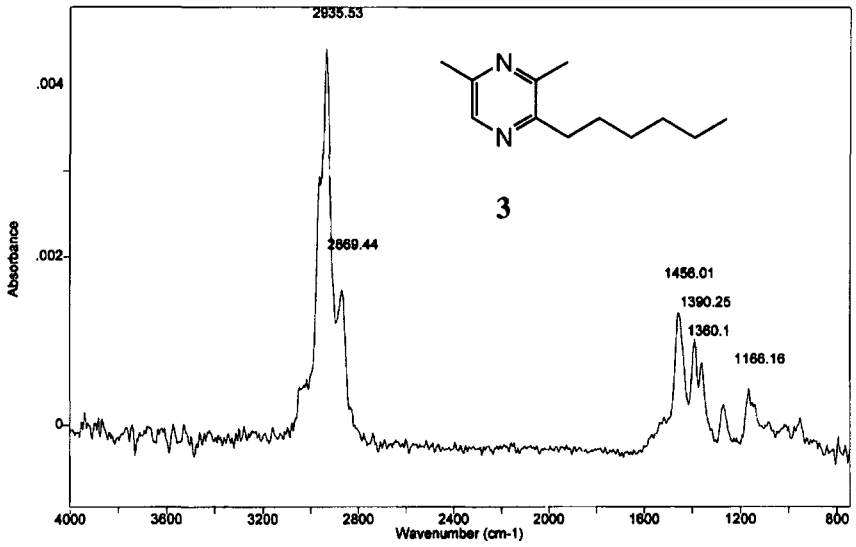

FIG. 1. Continued.

standards, the FTIR spectra of some known, isomeric dimethylaklylpyrazines might permit assignment of the geometry of **1**, **2**, and **3**. Since the three possible isomers of dimethylpropylpyrazine (3,5-dimethyl-2-propylpyrazine, **4**; 2,3-dimethyl-5-propylpyrazine, **5**; and 2,5-dimethyl-3-propylpyrazine, **6**) were commercially available, their FTIR spectra were obtained (Figure 2). While the FTIR spectra of **4**, **5**, and **6** are similar, they are clearly distinguishable. The fingerprint region retains the sample profile for **1–4**, but is quite different for **5** and **6**, indicating a 3,5-dimethyl-2-alkyl substitution pattern for the homologous dimethylalkylpyrazines **1–3**. In particular, the doublet of absorptions at ~ 1360 and at ~ 1390 cm^{-1} that appears in their spectra is absent in the spectra of **5** and **6**. Interestingly, 2,6-dimethylpyrazine does not show these absorptions (Pouchert, 1989).

Further support for the assignment of structures **1**, **2**, and **3** was obtained when their gas chromatographic retention times were plotted against the number of carbon atoms in the side chain. A linear correlation was found that intersected the retention time of **4** (Figure 3). This result is typical for a homologous series of compounds. In addition, the retention time of **4**, which is distinct from those of **5** and **6**, matched that of the trace amounts (not enough for FTIR analysis) of the dimethyl-propylpyrazine detected in the ants. Previous studies of ant pyrazines from the mandibular gland used only comparison of gas chromatographic retention times to assign the structure of these compounds. GC-FTIR

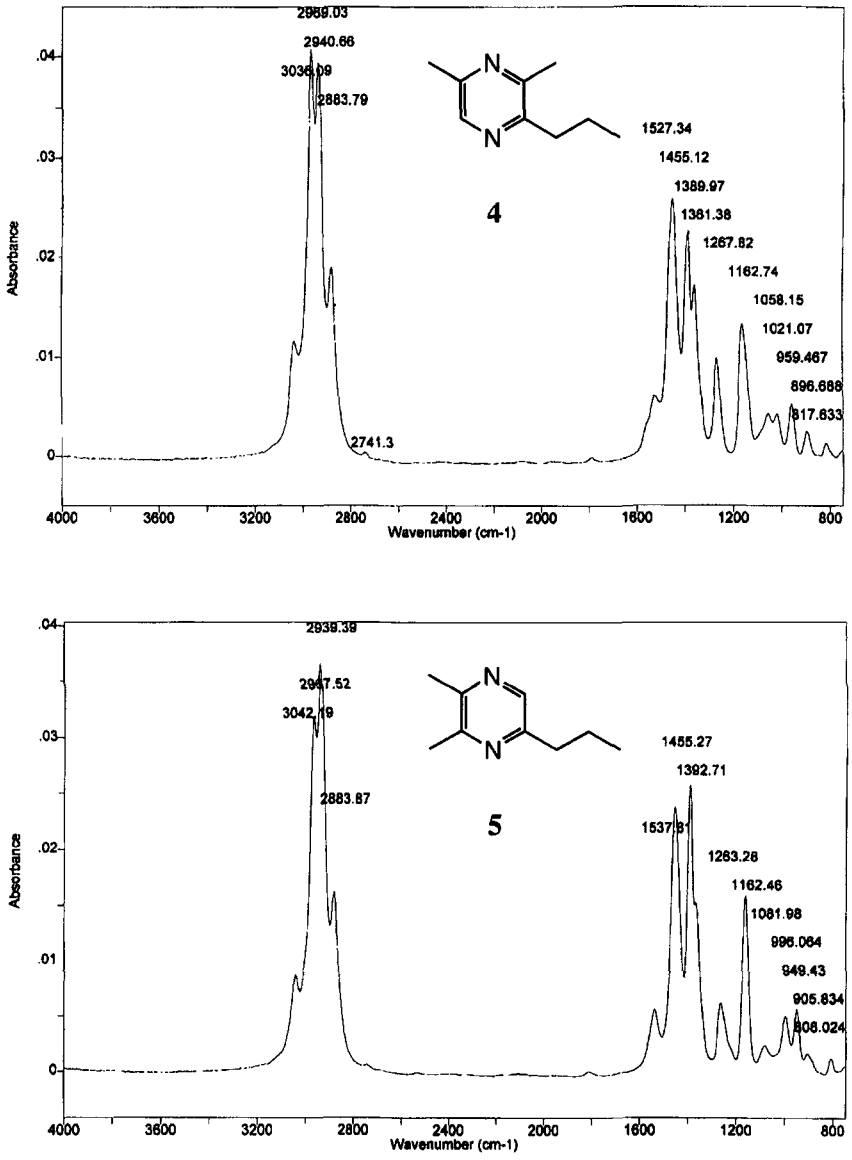

FIG. 2. GC-FTIR spectra of the dimethylpropylpyrazines **4**, **5**, and **6**.

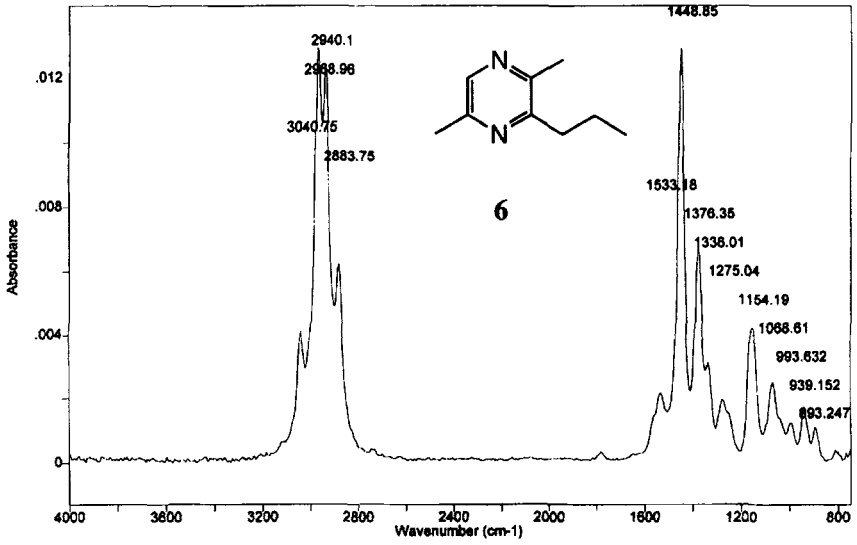

FIG. 2. Continued.

introduces another dimension, not only GC retention times but also FTIR spectra. Thus the comparisons are greatly strengthened, and the availability of all the isomers is not required.

Calibrated gas chromatograms showed that whereas the heads of unmated workers contained ca. 50–100 μg of total pyrazines, those of the gamergates contained only 0.5–1 μg of total pyrazines. Additionally, since 91 individuals were analyzed separately, the proportions of pyrazines in gamergates and nonmated workers were compared by t test using separate variances. Significant differences between workers and reproductives in average pyrazine content per ant were observed for **2** and **3**, but not for **1**. The hypothesis that workers and reproductives contain a different proportion of these compounds is supported at the $\alpha = 0.5$ significance level (Table 1).

Although *Streblognathus* and *Dinoponera* spp. both reproduce with monogamergates, the species in these two related genera (Carpenter, 1930; Haskins and Zahl, 1971) exhibit some common features as well as salient differences in terms of the exocrinological characteristics of their mandibular gland secretions. Gamergates and unmated workers of *S. aethiopicus*, as described here, produce 3,5-dimethyl-2-alkylpyrazines in their mandibular glands, whereas workers of two species of *Dinoponera*, *D. grandis* (Herman et al., 1984) and *D. australis* (Oldham et al., 1994), synthesize 2,5-dimethylpyrazine-3-alkylpyrazines in these exocrine structures. In both *Dinoponera* species the alkyl group is a branched

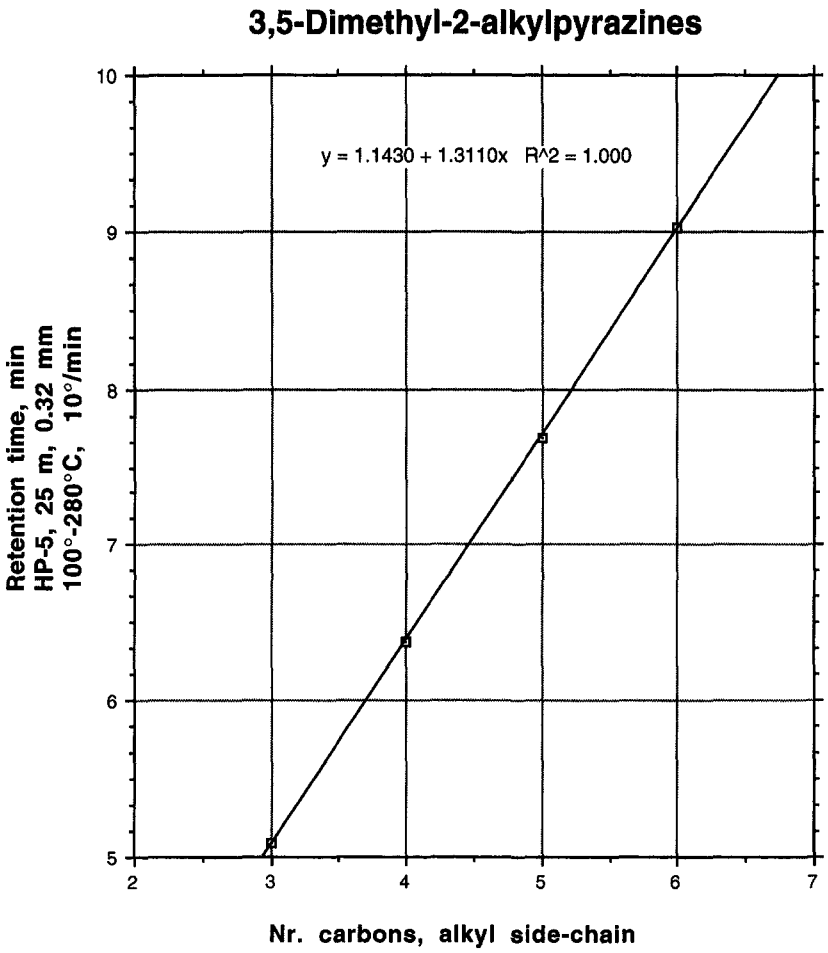

FIG. 3. Correlation of gas chromatographic retention time with alkyl side-chain carbon number.

isobutyl moiety in contrast to *S. aethiopicus*, where the alkyl group is an unbranched *n*-butyl, *n*-pentyl, or *n*-hexyl chain.

The mandibular gland secretions of the gamergates of *S. aesthiopicus* are distinctive in containing only about 0.5–1 μg of pyrazines, whereas the unmated workers contain 50–100 μg of these compounds. This is also the case for gamergates of *D. australis*, which produce about 0.1 μg of pyrazines, in contrast to the workers, which generate about 20 μg/worker (Oldham et al., 1994).

TABLE 1. *t* TEST USING SEPARATE VARIANCES TO TEST HYPOTHESIS THAT NOMINATED *S. aethiopicus* WORKERS HAVE DIFFERENT PROPORTIONS OF EACH PYRAZINE THAN GAMERGATES

Pyrazine	Pyrazine content (%)		
	1	2	3
Nonmated worker (mean ± SD)	50.78 ± 13.24	22.06 ± 10.13	26.9 ± 8.24
Gamergate (mean ± SD)	53.91 ± 7.74	13.86 ± 7.21	30.82 ± 6.01
t test (probability of H_0)	0.16	<0.001	0.01
$P < 0.05$?	No	Yes	Yes

Mating by workers of both *Streblognathus* and *Dinoponera* species obviously results in considerable inhibition in the biosynthesis of pyrazines compared to workers. This may indicate that the production of large amounts of pyrazines is not highly adaptive for gamergates, in contrast to the unmated workers. Since *Streblognathus* and *Dinoponera* are the only genera in a small complex of monogynous ponerine ants, it is worth noting that the unmated *S. aethiopicus* workers produce considerably more pyrazines than *Dinoponera* workers (50–100 μg vs. 5–48 μg). At the colony level, this disparity in the total quantity of these compounds is reinforced by the fact that colonies of the former contain more than 40 workers while colonies of *D. australis* average only 13 workers per colony.

Significantly, major qualitative differences distinguish the pyrazines produced by gamergates and workers of *D. australis* (Oldham et al., 1994). About 98% of the gamergate secretion is made up of a single compound, 2,5-dimethyl-3-(3′-methylbutyl)pyrazine, whereas the worker secretions contain five compounds that are quantitatively significant. By contrast, the same three pyrazines, **1**, **2**, and **3**, are produced by both gamergates and workers of *S. aethiopicus*. Thus, while gamergates of *D. australis* produce an essentially pure mandibular secretion in contrast to their unmated workers, gamergates of *S. aethiopicus* mirror their workers by producing a secretion that is qualitatively the same as that of unmated individuals.

Finally, it seems appropriate to discuss the possible function of the pyrazines produced by these species. We have no information on the role of these pyrazines in the biology of *S. aethiopicus*, and the same is true for *Dinoponera* (Oldham et al., 1994). Although ponerines generally utilize these compounds as releasers of alarm behavior (Wheeler and Blum, 1973; Longhurst et al., 1978), workers of neither *D. grandis* nor *D. australis* exhibit any obvious reac-

tion to crushed mandibular glands or neat compounds (Hermann et al., 1984; Oldham et al., 1994). This is also true for an unrelated ponerine, *Paraponera clavata* (Hermann et al., 1984). It may be that chemical communication is less highly evolved in these primitive ponerines vis-à-vis more advanced taxa. It will not prove surprising if these pyrazines play allomonal roles against a variety of competitors, a hypothesis that is readily amenable to experimental examination.

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ELICITORS OF PLANT DEFENSIVE SYSTEMS REDUCE INSECT DENSITIES AND DISEASE INCIDENCE[1]

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Abstract—Some elicitors of plant defensive systems can induce biochemical changes that enable the plant to reduce disease incidence; however, little is known about the effect of these induced responses on insect herbivores. We approached this problem using exogenous field applications of several abiotic elicitors of defensive systems in tomatoes (*Lycopersicon esculentum*), and evaluated the ability of the elicitors [benzo(1,2,3)thiadiazole-7-carbothioic acid (*S*)-methyl ester (BTH, Actigard); Probenazole; chitosan; salicylic acid; KeyPlex 350; KeyPlex DP2; and KeyPlex DP3] to reduce pest densities and to provide cross-resistance against various insect herbivores and pathogens. Only BTH provided cross-resistance and significantly reduced the incidence of bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*), early blight (*Alternaria solani*), leaf mold (*Fulvia fulva*), and leafminer larval densities (*Liriomyza* spp.). The effects on leafminer larval densities were more pronounced during the early stages of plant development. A trend of reduced densities of whiteflies (*Bemisia argentifolii*) and powdery mildew (*Oidium* sp.), although not significant, was also found on the BTH-treated plants. Other elicitors had no significant effect on insect populations, but Probenazole and KeyPlex 350 significantly reduced bacterial spot and early blight incidence. The antiherbivore effects of BTH on leafminers was confirmed in a laboratory two-choice experiment. Adult leafminers preferred untreated plants to the BTH-treated tomatoes as ovipositioning host plants, generally corresponding

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with larval performance. BTH induced high levels of pathogenesis-related proteins in tomato plants including peroxidase, lysozymes, chitinase, and β-1,3-glucanases. The possible cross-resistance role of these proteins is discussed. The demonstration that exogenous induction of plant defensive systems in the field can result in lower damage caused by various pathogens and insects, supports the hypothesis that plant defensive systems may be general.

Key Words—Elicitor, induced response, leafminers, pathogenesis-related protein, plant defense, tomatoes, whitefly.

INTRODUCTION

Plant pathogenesis-related (PR) proteins have been defined as those proteins that are induced and newly expressed in pathological or related situations (van Loon et al., 1994). This definition includes proteins that are induced as a result of phytophagous insects and other herbivore attacks. PR proteins such as chitinases and β-1,3-glucanases can degrade the cell walls of some phytopathogens and consequently may play a part in the host plant's defensive system (Dixon et al., 1994; Graham and Sticklen, 1994). PR proteins are induced by abiotic substances such as ethylene, chitin, chitosan, etc., and their evaluation may also raise the resistance level to plant pests (Dixon et al., 1994; Graham and Sticklen, 1994).

Insect herbivores often induce PR proteins in their plant hosts as a result of feeding (Stout et al., 1994). Mayer et al. (1995) have reported that acidic chitinases in citrus roots are induced as a result of West Indies sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* Li) larvae feeding on the roots and that induction is dependent on rootstock variety.

Various insects feeding on tomatoes induce PR proteins. This may indicate that PR proteins play a role in a plant's defense against insect attacks. Stout et al. (1994) have reported that polyphenol oxidase, peroxidase, lipoxygenase, and two proteinase inhibitors are elevated in tomato plants fed on by tomato fruitworm [*Helicoverpà zea* (Boddie)], leafminer [*Liriomyza trifolii* (Burgess)], and russet mites [*Aculops lycopersici* (Massee)]. In addition, feeding by the silverleaf whitefly (*Bemisia argentifolii* Bellows & Perring) has been shown to induce chitinases, β-1,3-glucanases, and peroxidases in tomatoes (Mayer et al., 1996).

Induction of defensive systems in plants can be achieved and enhanced during abiotic elicitors. These are compounds that act as signals that stimulate the synthesis of natural products, phytoalexins, and PR proteins that reduce pest damage (Benhamou and Theriault, 1992; Ebel and Cosio, 1994). If an elicitor induces a general defense response in the host plant, we would expect to detect effects on a variety of pests, i.e., pathogens and insects. Little is known about the effect of PR proteins on insect herbivores and whether or not exogenic elicitors can provide cross-resistance.

In this study, we examined the ability of several abiotic elicitiors to reduce disease incidence and insect herbivore densities in the field. In controlled laboratory experiments, we characterized the induction of PR proteins by the most promising elicitor and confirmed its anti-herbivore effects on the preference and performance of leaf-mining insects.

METHODS AND MATERIALS

Culture Procedure and Field Plot Experimental Design. Six-week-old Agriset tomatoes (Speedling Inc., Bushnell, Florida) were transplanted on March 26, 1996, at the University of Florida, Agricultural Research and Education Center, Ft. Pierce, Florida. Raised beds (15 cm high, 1.06 m wide) were spaced at 2.13-m centers. Plants were transplanted 0.6 m apart in the center of each bed. Beds were covered with black polyethylene mulch and watered with subsurface irrigation. Diamond R-7315 fertilizer (Diamond R Fertilizer Co., Winter Garden, Florida) was incorporated into the beds at a rate of 50-200-50 kg/acre of N-P-K, respectively. In addition, Diamond R 7314 was applied to the bed surface at rates of 199-299-498 kg/acre N-P-K. An additional single foliar fertilization of 20-20-20 N-P-K was applied three weeks after transplanting at a rate of 2.27 kg/379 liters. Two beds, each with 12 plants, were considered as plots. Plots were arranged as randomized complete block replicates with five replicates. Buffer zones (3.6 m) were maintained between plots. No pesticides were used on the plots at any time during the experiment. However, buffer zones were sprayed twice with the herbicide Gramoxone (ICI, Wilmington, Delaware), at the rate of 0.7 liters/acre.

Chemical Treatments. Tomatoes were treated with six different materials as follows: Actigard [benzo(1,2,3)thiadiazole-7-carbothioic acid(*S*)-methyl ester (BTH) (Novartis Crop Protection, Inc., Greensboro, North Carolina] was applied every three weeks (total four applications; Figure 1a). Foliar applications of BTH [1.85 g ai (active ingredient)/3.8 liter water] were made using a hand

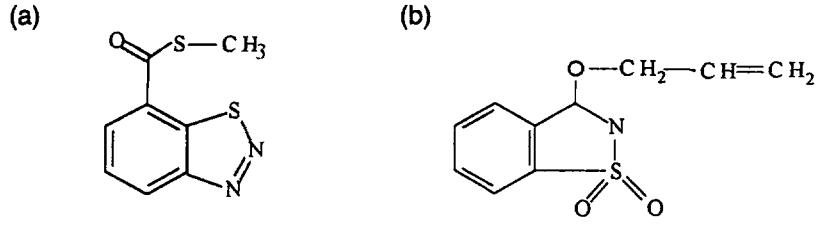

FIG. 1. Structures of (a) Actigard [benzo(1,2,3)thiadiazole-7-carbothioic acid (*S*)-methyl ester] BTH and (b) Probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide).

sprayer. The nutritional products, KeyPlex 350, KeyPlex DP2, and KeyPlex DP3 (Morse Enterprises, Miami, Florida) were applied to the soil every two weeks (six applications) at a rate of 50 ml (0.5%, v/v) per plant. A mixture of salicylic acid and chitosan was applied to the soil every two weeks (total of six applications) at rates of 50 ml (0.0015% + 0.00003% w/v respectively) per plant. Probenazole [3-allyloxy-1,2-benzisothiazole-1,1-dioxide (Meiji Seika Kaisha, Ltd., Tokyo, Japan)] was mixed with the soil only once during transplanting at rates of 6 kg/acre (Figure 1b). Control plants did not receive any chemical treatments. Except for Probenazole, the first treatment was 24 hr before transplanting. When BTH was applied, the remaining plants received water applications. Likewise, when soil applications of the other products were made, the BTH- and Probenazole-treated plants received 50 ml water. The KeyPlex 350, and the salicylic acid + chitosan mixture treatments ended with four replicates.

Monitoring of Insect Populations. The total number of mines of *Liriomyza* spp. (Diptera: Agromyzidae) on the adaxial surface of the three terminal leaflets of the seventh leaf from the top of the plant were counted, as suggested by Schuster and Beck (1992). Ten leaves per plot (randomly selected) were counted every week. Whitefly eggs and nymphs were counted on the terminal trifoliate using a stereomicroscope. Because whitefly densities were low, we examined the entire abaxial surface of these leaflets. Trifoliate area was measured after counting, using a leaf area meter (LI 3000, Lambda Instruments Corp., Lincoln, Nebraska).

Yellow sticky traps (7.6 × 12.7 cm; Olson Products Inc., Medina, Ohio) were placed in the center of each plot between beds at 50.8 cm height. Adult *B. argentifolii* were counted weekly. Insect populations (adults and immatures separately) for each sampling date were subjected to two-way analysis of variance (Sokal and Rohlf, 1981) with date and treatments as main effects. Data analyses followed Student-Newman-Keuls test (S-N-K) at the 0.05 level of significance. A Pearson correlation analysis was used to examine the relationships between densities of immature whiteflies and adults that were counted on the yellow traps. Mean numbers of insects were subjected to square root transformation before analysis.

Monitoring Disease Incidence. We monitored the incidence of several diseases including *Xanthomonas campestris* pv. *vesicatoria* (Doidge) dye (bacterial spot), *Fulvia fulva* (Cooke), Cif. (leaf mold of tomato), *Alternaria solani* Sorauer (early blight), and *Oidium* sp. (powdery mildew of tomato). Readings of each disease were taken from all the plants in the field on June 17, 1996. The plants were manipulated so that stem and leaf surfaces could be viewed; thus, the disease ratings are on a whole-plant basis. Disease rankings were 0 = no symptoms observed; 1 = a few lesions present on less than 10% of foliage; 2 =

scattered lesions on up to 50% of the foliage; 3 = lesions present on most of the foliage; 4 = very high incidence of lesions on most foliage.

Laboratory Experiments with BTH. Because of the encouraging results of the BTH effect on leafminers (*Liriomyza* sp.) in the field trials (see results), we tested BTH efficacy in controlled laboratory experiments. One-month-old Agriset tomatoes (Speedling Inc.) were maintained in a greenhouse in 5.7-cm-diameter pots with Metro Mix 500 growing medium (Grace Sierra, California). Half the plants ($N = 14$) were treated with three foliar applications (every two weeks) of BTH (1.85 g ai/3.8 liters water), as in the field experiments. Control plants were sprayed with water. Four days after the last application, 14 pairs of control and BTH-treated plants were used for choice and performance experiments with leafminers. Each pair of plants was placed in a 15.2-liter bucket sealed with cellophane. Six unsexed, newly emerged, adult leafminers [*Liriomyza trifolii* (Burgess)] were introduced into each bucket. Adults were allowed to feed and oviposit for 24 hr and then removed. Plants were maintained in a controlled atmosphere room (50% relative humidity, 28°C) throughout the experiment. One week later, whole plants were placed horizontally in a plastic dish, and puparia were collected. The experiment was terminated 13 days after the removal of the adults. Adult leafminer host preference was measured by counting the number of mines, oviposition, and feeding punctures per plant and per leaf area. Survival (based on collected puparia) was calculated as an indication of larval performance. Leafminers were from a culture initiated in 1983 and maintained on cowpea, *Vigna sinensis* (*Stickm.*). The data were analyzed using the paired comparison t test (Sokal and Rohlf, 1981). The number of mines, oviposition, and feeding punctures, as well as the number of infested leaves and leaflets, were subjected to square root transformation before analysis.

Another batch of 28 tomato plants (half treated with BTH), maintained and treated similarly to those used for the leafminer experiment, was used to determine the biochemical effects of BTH on tomatoes. Leaves were randomly harvested when the leafminer experiment was terminated and kept in the freezer (−20°C) until analysis. Foliar protein was measured by the Bradford (1976) procedure. The induction of several PR proteins (chitinase, peroxidase, β-1,3-glucanase, and lysozyme) was measured with enzymatic and immunological methods described previously (Mayer et al., 1995).

RESULTS

Leafminer Populations in Field Experiments. Leafminer populations increased relatively fast on the young tomato plants. Leafminer larval population densities increased rapidly and reached a peak four weeks after transplanting, followed by a gradual but consistent reduction throughout the experiment (Figure

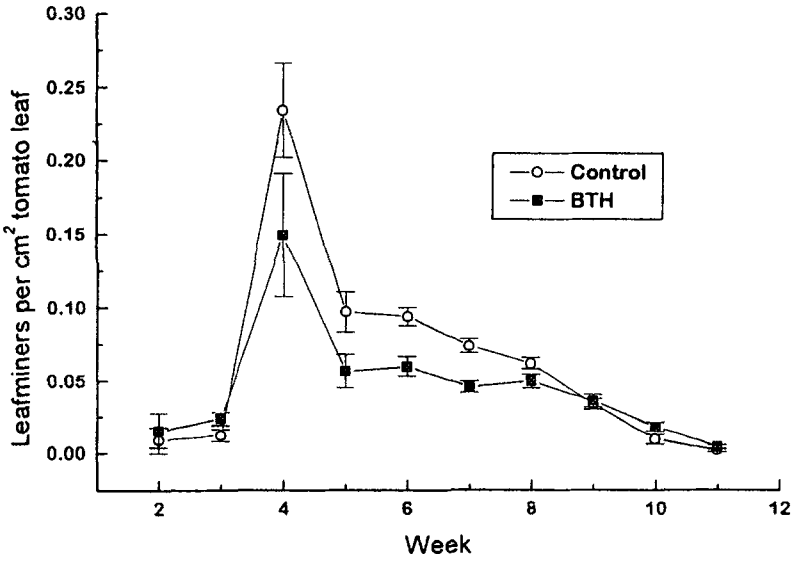

FIG. 2. Effects of Actigard (BTH) applications on larval leafminer populations in tomatoes. BTH significantly reduced leafminer density in weeks 4, 5, and 6 after transplanting ($F_{6,26} = 2.2$, $P < 0.05$). Other elicitors had no effect on leafminers at any time (S-N-K, $P > 0.05$) and therefore are not shown. Error bars indicate 1 SE.

2). Only in BTH-treated plants and only during the peak of the population density did we find a significant reduction in the number of leafminers per trifoliate ($F_{6,26} = 2.2$, $P < 0.05$). Compared with the control plants, BTH-treated plants had 34.7% fewer leafminers per square centimeter leaf area four weeks after transplanting, 40% fewer leafminers at five weeks, and 33% fewer leafminers at six weeks (Figure 2). Other treatments did not significantly reduce leafminer larval populations at any time (S-N-K, $P > 0.05$).

Whitefly Population in Field Experiments. In contrast to leafminers, whitefly populations took more than two months to build up. None of the treatments was found to be statistically significant in reducing whitefly egg (Figure 3), nymph (Figure 4), or adult (Figure 5) densities. However, two trends were observed. First, control plants supported more whiteflies than did any of the treatment groups. Second, BTH-treated plants had consistently lower whitefly adult, nymph, and egg densities compared with other treatments and the control. We found a strong correlation between adult whiteflies caught on traps and whitefly nymphs ($r = 0.69$, $P < 0.01$) and eggs ($r = 0.61$, $P < 0.01$) counted on the leaves.

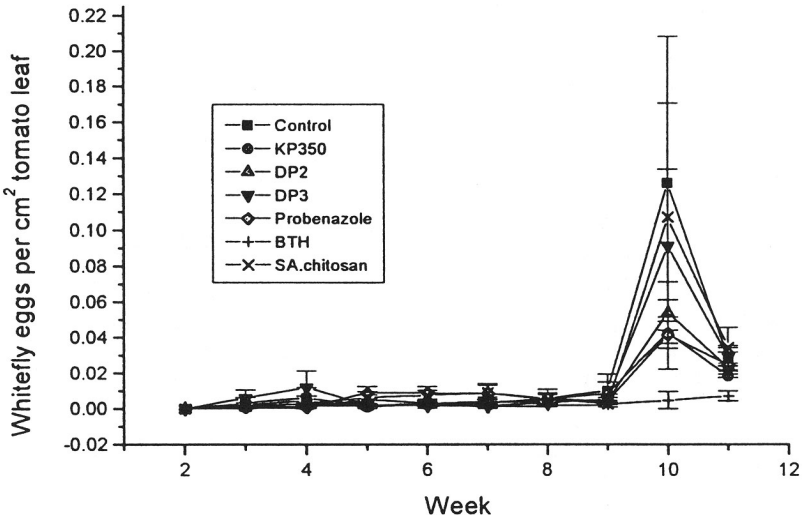

FIG. 3. Effects of elicitor applications on whitefly egg densities in tomatoes. None of the treatments had significant effect at any time ($F_{6,26}$ = 1.58, NS). Error bars indicate 1 SE.

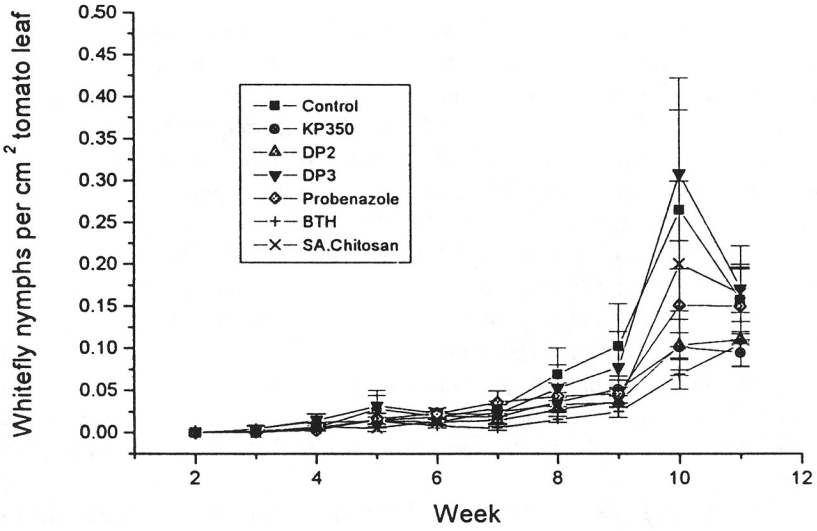

FIG. 4. Effects of elicitor applications on whitefly nymph densities in tomatoes. None of the treatments had significant effect at any time ($F_{6,26}$ = 1.57, NS). Error bars indicate 1 SE.

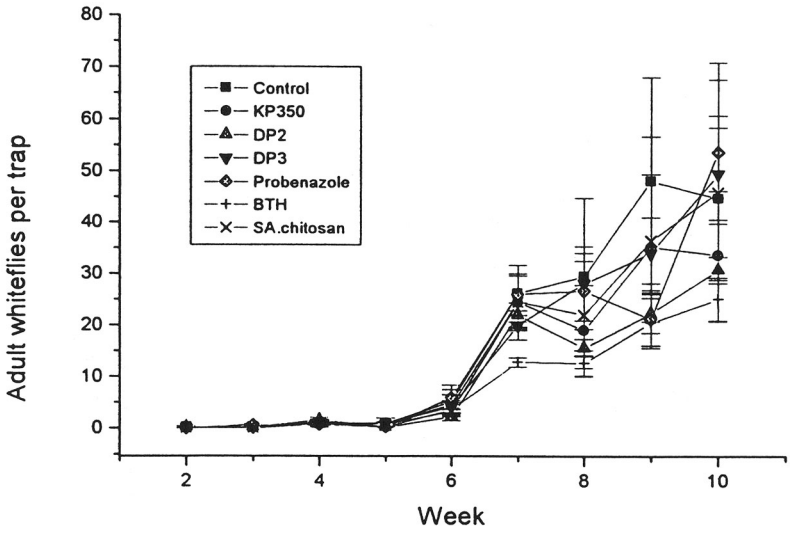

FIG. 5. Effects of elicitor applications on whitefly adult populations in tomatoes. None of the treatments had significant effect at any time ($F_{6,26} = 1.08$, NS). Error bars indicate 1 SE.

Disease Incidence in Field Experiments. Disease incidence for each of the treatments is given in Table 1. There were no significant differences for any of the treatments on powdery mildew incidence ($F_{6,26} = 1.08$, NS). The incidence of bacterial spot ($F_{6,26} = 11.1$, $P < 0.01$) and early blight ($F_{6,26} = 6.9$, $P < 0.01$) was significantly different from controls for Probenazole, KeyPlex 350, and BTH treatments. Only BTH-treated plants had a significantly lower incidence of leaf mold ($F_{6,26} = 4.3$, $P < 0.01$).

Induction of PR Proteins by BTH. Applications of BTH to tomato plants significantly induced PR proteins (Table 2). Foliar levels of chitinase, β-1,3-glucanase, and lysozyme were about two fold higher in the BTH-treated plants compared to the controls. Induction of peroxidase was less pronounced but highly significant. Significantly different levels of total proteins were not found in the BTH-treated plants. Unlike the field experiments, some phytotoxic effects were observed in the laboratory experiments where BTH-treated plants appeared to be smaller.

Effect of BTH on Leafminers in Laboratory Experiments. The effect of BTH on leafminer preference and performance is summarized in Table 3. Adult female leafminers recognized BTH-treated plants as less preferable hosts for oviposition. The number of eggs laid and mine densities were lower on the

TABLE 1. DISEASE INCIDENCE IN TOMATOES TREATED WITH DIFFERENT ELICITORS

Treatment	Disease rating[a]			
	Powdery mildew (*Oidium* sp.)	Bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*)	Early blight (*Alternaria solani*)	Leaf mold (*Fulvia fulva*)
Control	0.33a	2.47a	2.24a	2.08a
Salicylic acid + chitosan	0.69a	2.54a	2.23a	1.59a
KeyPlex 350	0.80a	1.70c	1.61b	1.77a
KeyPlex DP2	0.60a	2.12ab	2.62a	1.99a
KeyPlex DP3	0.45a	2.42a	2.16a	2.06a
Probenazole	0.20a	1.78b	1.53b	1.99a
BTH	0.28a	1.31c	1.50b	0.99b

[a]Numbers followed by like letters within columns indicate no significant differences.

TABLE 2. EFFECT OF BTH ON INDUCTION OF DEFENSIVE PROTEINS IN TOMATOES[a]

Treatment	Protein (mg/g tissue)	Peroxidase (ΔA_{510}/min/g tissue)	Lysozyme (ΔA_{510}/min/g tissue)	Chitinase (ΔA_{510}/min/g tissue)	β-1,3-Glucanase (mmol Glc/min/g tissue)
Control	0.5 ± 0.02	29.4 ± 1.4	124.5 ± 12.5	1.7 ± 0.1	0.39 ± 0.02
BTH	0.65 ± 0.06	36.9 ± 2	317.9 ± 32.2	3.3 ± 0.2	0.84 ± 0.07
t	1.7 NS	3.02*	5.5*	5.7*	5.7*

[a]Values are mean ± SE; NS, nonsignificant; *$P < 0.01$; each treatment with 14 replicates.

BTH-treated plants. The incidence of adult feeding on BTH-treated plants was not significant. BTH-treatments reduced the number of leaves and leaflets mined by approximately half. Survival of leafminer larvae was not lower on the BTH-treated plants.

DISCUSSION

Mayer et al. (1995, 1996) have suggested that plant defensive proteins might be used to control some insect pests. Chitosan and salicylic acid are known

TABLE 3. EFFECT OF BTH ON ADULT LEAFMINER HOST SELECTION AND LARVAL SURVIVAL (PERFORMANCE)[a]

Treatment	Mines/plant	Mines/cm^2	Eggs/cm^2	Feeding/cm^2	Leaves infested	Leaflet infested	Larval survival (P)
Control	3.2 ± 0.6	0.15 ± 0.03	0.17 ± 0.04	0.7 ± 0.2	2.4 ± 0.1	5.5 ± 1.2	0.22 ± 0.05
BTH	1.6 ± 0.5	0.08 ± 0.02	0.09 ± 0.02	0.53 ± 0.19	1.1 ± 0.34	2.5 ± 0.8	0.16 ± 0.05
Paired t	3.3*	2.2*	2.3*	0.6 NS	2.9*	3.2**	1.4 NS

Survival rate is the ratio between the number of eggs laid and puparia collected at the end of the experiment. The experiment was designed and analyzed as paired comparison ($n = 14$).

[a] Values presented as mean and SE; *$P < 0.05$; **$P < 0.01$; NS, nonsignificant.

elicitors of PR proteins in many different types of plants (Benhamou and Theriault, 1992; Ebel and Cosio, 1994; Klessig and Malamy, 1994), and salicylic acid has been associated with insect resistance in rice plants (Ishii et al., 1962). Probenazole is an elicitor that is used to reduce fungal diseases in rice (Midoh and Iwata, 1996), and BTH is marketed as an activator of plant defensive systems (Ciba-Geigy, 1995). KeyPlex 350 is a micronutrient preparation that is believed to be an elicitor (G. Butler, Morse Enterprises, personal communication) and the KeyPlex DP series are test products that use KeyPlex 350-based materials.

We have demonstrated that applications of BTH to tomato plants induced several known PR proteins and that applications effectively reduced the incidence of diseases and populations of insect herbivores. Tomato resistance is based on a complex of defensive systems that include PR proteins, proteinase inhibitors, polyphenol oxidases, and phytoalexins (Duffey and Stout, 1996). The induction of some of the proteins measured is thought to have multiple negative effects on pathogens and insect pests. Chitinase degrades chitin, a major component of pathogen cell walls (Graham and Sticklen, 1994). Chitinase may severely affect insects by damaging chitin-based structures such as the peritrophic membrane that provides a physical barrier to ingested pathogens and other substances that pose a hazard to the insect. Chitinases can also act as α-amylase inhibitors and interfere with digestion of plant parts (Ary et al., 1989). Induction of chitinase activity may interfere with insect development, feeding and growth, facilitate microbial infection, and finally cause death (Shapiro et al., 1987; Wang et al., 1996). Recently, Wang et al. (1996) reported 100% larval mortality of the grain beetle, *Oryzaephilis mercator*, six days after feeding on 2% chitinase obtained from transgenic tobacco. Lysozymes are defensive enzymes that protect plants against bacterial pathogens (Boller et al., 1983). These hydrolases act on murein, a peptide glycan cell wall component of bacteria. Their exact impact on insects is unknown; however, they may affect intestinal flora during ingestion and digestion and subsequently affect development.

Peroxidases are induced in tomato plants following pathogen and insect damage. Peroxidases are involved in production and polymerization of phenolics, lignification, and hypersensitive responses, limiting the possibility of disease spread (Bowles, 1990). Peroxidases also have negative effects on food digestibility and protein availability to herbivorous insects (Duffey and Felton, 1991; Duffey and Stout, 1996). β-1,3-Glucanase hydrolyzes β-1,3-glucans, which are major components of the surface structure and cell walls of many microbial and fungal pathogens (Bowles, 1990) but have no apparent effect on insects.

Whether induced plant responses to pathogen and insect pests are specific or general and whether or not cross-immunization actually occurs is currently

under debate (Karban and Myers, 1989; Apriyanto and Potter, 1990; Baldwin, 1994; Hatcher, 1995). However, as pointed out by Ebel and Cosio (1994), some biotic elicitors are race-specific, providing limited defense against pathogens. Other elicitors are more general, inducing a wide range of defenses against herbivores and pathogens. Our results support this view, suggesting that the degree of specificity of the defensive response will be dependent on the nature of elicitors used. Probenazole and the Keyplex-based elicitors that were tested in the field successfully reduced disease incidence but had no effect on the insects. Induced responses resulting from BTH applications were useful in providing general protection against pests belonging to different phyla. There is a growing body of evidence that induced defenses are general and, therefore, can provide resistance across phyla. For example, Karban et al. (1987) found that induced responses in cotton following fungi and spider mite infestations had reciprocal negative effects on the two pests. Infection of cotton with *Bacillus megaterium* and *B. cereus* induced systemic production of phytochemicals that affected boll weevils (Benedict and Chang, 1991). McIntyre et al. (1981) demonstrated that TMV infection of tobacco induced general resistance against a variety of pathogens and insect herbivores. Inoculation of soybeans with extracts of *Phytophthora megasperma* induced phytoalexin production that deterred feeding by the Mexican bean beetle (Kogan and Fischer, 1991).

Leafminer density and larval performance may be affected by the level of foliar proteins (Minkenberg and Ottenheim, 1990). We found a nonsignificant trend of increased total protein content in the BTH-treated plants. Thus, total protein (i.e., nitrogen) levels in this experimental system could not explain leafminer preference and performance. Regardless of the mechanism(s) involved, our results indicate that female leafminers have the ability to discriminate between favorable and less favorable host plants for larval development, based on the level of induced defensive substances, in addition to their response to total foliar protein levels (Minkenberg and Ottenheim, 1990).

Several factors may explain the lack of effect of BTH on whiteflies compared with its effect on the leafminers. First, throughout the experiment whitefly populations remained low despite gradual increases in populations towards the end of the growing season, perhaps preventing sensitive statistical detection of treatment effects. Secondly, unlike whiteflies, leafminer populations increased sharply soon after transplanting. Indeed, at this stage, we found significant reductions in mine densities. Although induced resistance in tomatoes may be detected in all plant stages, it is most pronounced in young seedlings (Alarcon and Malone, 1995). Thaler et al. (1996) showed that field applications of jasmonic acid induced several defensive compounds in tomato seedlings. Our field experiments provide additional evidence that induced defense responses in tomatoes are more effective in young plants to a level that can reduce insect herbivore densities. In addition, whiteflies are phloem-feeders (Cohen et al., 1996), while

L. trifolii larvae feed in the palisade mesophyll (Parrella, 1987). It has long been suggested that phloem-feeders are less exposed to plant chemical defense systems. Most plant toxins and other defensive compounds including chitinase (Wang et al., 1996) and proteinase inhibitors (Walker-Simmons and Ryan, 1977) are thought to be stored in intracytoplasmic vacuoles in parenchyma and epidermal cells (e.g., Rosenheim et al., 1996, and references therein). Vascular tissue should not be considered as defenseless, but it is possible that BTH-induced substances are less effective against phloem-feeders such as whiteflies. Finally, one cannot rule out the possibility that whiteflies may be less susceptible to the effects of PR proteins.

It appears that plant defensive systems can be used to enhance insect resistance in plants. Elicitors can be used to activate plant defensive systems at desired times; however, generally they should be applied prior to having a pest problem so that the plant will have the best opportunity for resisting pests (Ciba-Ceigy, 1995). Elicitors will probably not be effective in all plants since defensive systems vary with plant variety. Neither will they be effective against all insects since insects vary widely in their abilities in overcoming plant defensive systems. Considerable research will have to conducted to determine how best to use plant defense responses in insect control strategies.

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DISRUPTION OF SECONDARY ATTRACTION OF THE SPRUCE BEETLE, *Dendroctonus rufipennis*, BY PHEROMONES OF TWO SYMPATRIC SPECIES

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Abstract—Capture of spruce beetles, *Dendroctonus rufipennis*, in multiple-funnel traps baited with frontalin and α-pinene was reduced by up to 42% in the presence of synthetic (+)-*exo*- and (+)-*endo*-brevicomin, aggregation pheromones of the sympatric species *Dryocoetes affaber*. (±)-*endo*-Brevicomin was inhibitory to spruce beetles in two experiments and (±)-*exo*-brevicomin was inhibitory in one experiment, reducing spruce beetle trap catches by up to 87% and 75%, respectively. Spruce beetle trap catches were also reduced by 85% by (±)- or (+)-ipsdienol, but not by (−)-ipsdienol. *Ips tridens*, a second sympatric species, produces both enantiomers of ipsdienol in its pheromone blend. Responses by *D. affaber* to its own pheromone were significantly enhanced by addition of the spruce beetle lure. Enantiospecific pheromones of secondary competing species, or less costly racemic substitutes, may be useful for managing spruce beetles using competitive displacement or exclusion. Baiting susceptible hosts with pheromones of secondary species may enhance attack by secondary species, while partially repelling spruce beetles.

Key Words—*Dendroctonus rufipennis*, *Ips tridens*, *Dryocoetes affaber*, bark beetle, pheromone, inhibition, frontalin, α-pinene, ipsdienol, *cis*-verbenol, *exo*-brevicomin, *endo*-brevicomin, enantiomers.

INTRODUCTION

The spruce beetle, *Dendroctonus rufipennis* Kirby, is a major pest of Engelmann spruce, *Picea engelmannii* Parry, white spruce, *Picea glauca* (Moench) Voss,

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and their hybrids in British Columbia (Safranyik, 1988). The beetles preferentially breed in freshly fallen or weakened trees; however, if breeding material is abundant and environmental conditions are favorable, populations can build up to epidemic levels and healthy standing trees may be attacked, resulting in extensive outbreaks (Furniss and Carolin, 1975).

The spruce beetle is frequently associated with several species of secondary bark beetles, including *Ips tridens* Mannerheim, *Ips perturbatus* Eichhoff, *Dryocoetes affaber* Mannerheim, and *Polygraphus rufipennis* Kirby (Werner and Holsten, 1984). The most common secondary species in southern British Columbia are *I. tridens* and *D. affaber*. The secondary species are not considered to be economically important in British Columbia because they are incapable of killing healthy trees; they generally attack downed material or trees already overcome and killed by the spruce beetle. However, in Alaska *I. tridens* has recently been reported killing young white spruce following heavy thinning and pruning treatments and selective logging aimed to minimize spruce beetle-caused tree mortality and reduce fire hazard in sensitive areas (Holsten et al., 1996). Interspecific competition for breeding material and food by secondary bark beetles including *Scierus annectans* LeConte, *P. rufipennis*, and *Ips* spp., primarily *Ips pilifrons* Swaine, was shown to be a major cause of larval mortality for the spruce beetle in Colorado (McCambridge and Knight, 1972).

Competitive interactions may be exploited as a management tool if secondary species can be induced by semiochemicals to preempt a resource and outcompete or exclude a primary pest species (Payne and Richerson, 1985; Borden, 1989). Such an effect has been demonstrated experimentally for the pine engraver, *Ips pini* (Say), a secondary bark beetle capable of outcompeting the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major pest of lodgepole pine, *Pinus contorta* var *latifolia* Engelmann (Rankin and Borden, 1991; Safranyik et al., 1996).

The efficacy of competitive displacement or exclusion might be enhanced if the semiochemicals used to induce attack by secondary species were repellent to the primary pest species. Interruption of pheromonal responses by competing sympatric species has been observed for several scolytids. For instance, in California *I. pini* and the California fivespined ips, *Ips paraconfusus* Lanier, compete for breeding material in weakened or fallen ponderosa pine, *Pinus ponderosa* Laws. Attraction of beetles in each species to their conspecifics was reduced in the presence of heterospecifics (Birch and Wood, 1975). The aggregation pheromones ipsenol (2-methyl-6-methylene-7-octen-4-ol) and (+)-ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol), produced by *I. paraconfusus*, disrupted attraction of *I. pini* to logs containing conspecific males (Birch and Light, 1977; Birch et al., 1980). In addition, (−)-ipsdienol produced by *I. pini* inhibited responses of *I. paraconfusus* to conspecifics (Birch et al., 1980). Similarly, attraction of western pine beetles, *Dendroctonus brevicomis* LeConte, and

I. paraconfusus to ponderosa pine bolts infested with conspecifics was mutually inhibited in the presence of nearby bolts infested with beetles of the other species (Byers and Wood, 1980). A blend of ipsenol, ipsdienol, and *cis*-verbenol (*cis*-4,6,6-trimethylicyclo[3.1.1]hept-3-en-2-ol), the aggregation pheromone for *I. paraconfusus*, inhibited the response of *D. brevicomis* to its pheromones *exo*-brevicomin (*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) and frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) plus kairomonal myrcene (2-methyl-6-methylene-2,7-octadiene). Attraction of *I. paraconfusus* to its pheromones was unaffected by the aggregation pheromones of *D. brevicomis* but was reduced by the antiaggregation pheromone verbenone, 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one (Byers and Wood, 1980). In British Columbia, *I. pini* coexists with the mountain pine beetle and *Ips latidens* LeConte. Attraction of *I. pini* to synthetic ipsdienol and attacks on felled lodgepole pine were reduced by ipsenol, an aggregation pheromone for *I. latidens*, and verbenone, an antiaggregation pheromone for the mountain pine beetle (Borden et al., 1992; Devlin and Borden, 1995). Blends of antiaggregation pheromones and repellent synomones produced by sympatric competitors may be even more effective. For instance, verbenone and ipsdienol acted synergistically in inhibiting attraction by the western pine beetle (Paine and Hanlon, 1991).

Semiochemical disruptants for primary pest species, which also induce attack by secondary species, may enhance management efforts through simultaneous disruption and competitive displacement. For instance, treating loblolly pines, *Pinus taeda* L., with a blend of *exo*- and *endo*-brevicomin for disruption of the southern pine beetle, *Dendroctonus frontalis* Zimmerman, also resulted in colonization and competitive displacement of *D. frontalis* by *Ips avulsus* Eichhoff (Payne and Richerson 1985).

While the fate of behaviorally disrupted beetles is unknown, prolonged dispersal in search of suitable hosts would likely result in greatly reduced survival due to predation, desiccation, and metabolic exhaustion. In field tests to evaluate the efficacy of verbenone for disruption of *D. frontalis*, growth of treated infestation spots was completely halted with few or no additional trees being attacked (Payne and Billings 1989). Therefore, disrupted or displaced beetles would be unlikely to establish new attacks or infestations successfully.

Secondary scolytids may outcompete the primary tree-killing species by rapid larval development and utilization of phloem tissue (McCambridge and Knight, 1972; Rankin and Borden, 1991). Successful colonization of healthy vigorous trees by primary attacking species is often dependent on the establishment of mutualistic blue stain fungi, which colonize the phloem tissue and sapwood medullary parenchyma, inhibiting water conduction by the tree and reducing host resistance (Reynolds, 1992). In weakened or dead trees, selective pressures favor opportunistic secondary species that can exploit the available resources rapidly. Therefore, secondary species may be able to attack in nature

some days after initial attack by the primary species and still overcome their tree-killing rivals. It is possible that they exploit the pheromone of the primary species as an attractant alone or that the semiochemical blends of primary and secondary species interact in an additive or synergistic fashion in attracting the secondary species.

The commercial semiochemical lure for the spruce beetle consists of the pheromone frontalin (Gries et al., 1988) and the host kairomone α-pinene (2,6,6-trimethyl-bycyclo[3.1.1]hept-2-ene) (Furniss et al., 1976). The aggregation pheromone for *D. affaber* consists of a 1:2 mixture of (+)-*exo*- and (+)-*endo*-brevicomins (Camacho et al., 1994). *I. tridens* is attracted to a blend of (−)-*cis*-verbenol and (±)-ipsdienol (Moeck et al., 1985), two compounds that are produced, respectively, by males exposed to α-pinene vapors and by males boring into host logs (Poland, 1997). Enantiospecific pheromones are expensive, whereas racemic mixtures are cheaper and would be preferred for operational use if they were effective in eliciting appropriate beetle responses.

Our objectives were to test the hypotheses that: (1) known semiochemical attractants for *D. affaber* and *I. tridens* inhibit attraction of the spruce beetle to its pheromone; (2) racemic mixtures of *D. affaber* and *I. tridens* pheromones inhibit spruce beetle attraction to its pheromone as effectively as chirally pure blends; and (3) attraction of secondary species is enhanced when their pheromones are combined with that of the spruce beetle.

METHODS AND MATERIALS

Five field trapping experiments were conducted near Princeton, British Columbia, in mature stands composed of Engelmann spruce, lodgepole pine, and subalpine fir, *Abies lasiocarpa* (Hook) Nutt. All experiments utilized 12-unit funnel traps (Lindgren, 1983) set out in randomized complete blocks with at least 15 m between traps. Captured beetles were collected and stored in plastic bags at −18°C before they were sexed and counted. Ten replicates were set up initially for each experiment. For some experiments, all lures were collected after the first set of 10 replicates, and the experiments were repeated with an additional 10 replicates laid out in new randomized complete blocks, providing a total of 20 replicates in two time periods.

Semiochemical attractants for the spruce beetle, *I. tridens*, and *D. affaber* are summarized in Table 1. Spruce beetle funnel lures (Phero Tech Inc., Delta, British Columbia) were used as the attractive semiochemical bait for the spruce beetle. The lures consisted of a 1.5-ml Eppendorf tube containing α-pinene released at 50–80 mg/24 hr and a 400-μl tube containing frontalin released at 2 mg/24 hr.

The attractive lure for *I. tridens* consisted of three bubble caps each releas-

TABLE 1. SUMMARY OF SEMIOCHEMICALS AND THEIR BIOLOGICAL ACTIVITIES FOR *Dendroctonus rufipennis*, *Ips tridens*, and *Dryocoetes affaber*

Species	Semiochemical	Biological Activity	Reference
D. rufipennis	α-pinene	attractive	Furniss et al. (1976)
	frontalin	attractive	Gries et al. (1988)
D. affaber	(+)-*exo*-brevicomin	optimal attraction to 1:2 ratio of (+) enantiomers of *exo*- and *endo*-brevicomin	Camacho et al. (1994)
	(+)-*endo*-brevicomin		
	(−)-*exo*-brevicomin	no activity	
	(−)-*endo*-brevicomin	inhibits response to optimal blend	
I. tridens	(±)-ipsdienol	attractive	Moeck et al. (1985), Poland (1997)
	(−)-*cis*-verbenol	attractive	

ing (+)- and (−)-*cis*-verbenol in a 17:83 ratio at 0.6 mg/24 hr, and three bubble caps each releasing (±)-ipsdienol at 0.2 mg/24 hr. Chirally pure enantiomers of ipsdienol [97% pure (+)- or (−)-ipsdienol] were also released at 0.2 mg/24 hr from bubble caps. All release devices were obtained from Phero Tech.

Pheromone baits for *D. affaber* consisted of a 1:1 mixture of (±)-*exo*- and (+)-*endo*-brevicomin released at 0.2 mg/24 hr from glass capillary tubes (1.0 mm ID) sealed at one end and placed in perforated Eppendorf tubes (Stock et al., 1990). The presence of (−)-*exo*-brevicomin does not affect responses of *D. affaber*; however, (−)-*endo*-brevicomin is inhibitory. Therefore, (−)-*exo*-brevicomin and (+)-*endo*-brevicomin were used in a mixture that effectively delivered a 1:2 ratio of the (+) enantiomers (Camacho et al., 1994). Enantiomers of *exo*- and *endo*-brevicomin were released separately from devices prepared in the same manner as the *D. affaber* lures. Respective chemical and optical purities were: (+)-*exo*-brevicomin 98.1% and 94%; (−)-*exo*-brevicomin 97% and 95%; (+)-*endo*-brevicomin 98.8% and 90.15%; and (−)-*endo*-brevicomin 91% and 88%. Racemic *exo*- and *endo*-brevicomin (98% and 95.6% chemical purity, respectively) were obtained from Phero Tech. All chiral compounds were synthesized by B. D. Johnston (Department of Chemistry, Simon Fraser University) according to procedures developed by Johnston and Oehlschlager (1982).

Experiment 1 tested whether attraction of the spruce beetle to the spruce beetle lure is inhibited in the presence of the *D. affaber* pheromone. It comprised

10 replicates of four treatments: (1) unbaited control; (2) spruce beetle lure; (3) *D. affaber* lure; and (4) spruce beetle plus *D. affaber* lures.

Experiments 2 and 3 compared attraction of the spruce beetle to the different enantiomers of *exo-* and *endo*-brevicomin. Both comprised 20 replicates of five treatments. The treatments for experiment 2 were: (1) unbaited control; (2) spruce beetle lure; (3) spruce beetle lure plus (+)-*exo*-brevicomin; (4) spruce beetle lure plus (−)-*exo*-brevicomin; and (5) spruce beetle lure plus (±)-*exo*-brevicomin. Experiment 3 was identical to experiment 2 except that enantiomers of *endo*-brevicomin were used.

Experiment 4 compared (±)-*exo*- and (±)-*endo*-brevicomin alone and combined in 20 replicates of five treatments: (1) unbaited control; (2) spruce beetle lure; (3) spruce beetle lure plus (±)-*exo*-brevicomin; (4) spruce beetle lure plus (±)-*endo*-brevicomin; and (5) spruce beetle lure plus both (±)-*exo*- and (±)-*endo*-brevicomin.

Experiment 5 tested whether attraction of the spruce beetle to the spruce beetle lure is inhibited by the presence *I. tridens* pheromone. It was identical to experiment 1 except that *I. tridens* lures replaced *D. affaber* lures.

Experiment 6 tested whether the different enantiomers of ipsdienol disrupted attraction of the spruce beetle to the spruce beetle lure. It comprised 10 replicates of five treatments: (1) unbaited control; (2) spruce beetle lure; (3) spruce beetle lure plus (+)-ipsdienol; (4) spruce beetle lure plus (−)-ipsdienol; and (5) spruce beetle lure plus (±)-ipsdienol.

The numbers of beetles of each species captured were transformed by $\log_{10}(x + 1)$ to satisfy assumptions of normality and homogeneity of variances (Zar, 1984), and then analyzed by ANOVA for randomized complete block design treating replicates as blocks (SAS, 1990). An additional blocking factor for collection time was included for experiments with 20 replicates over two collection periods. The Ryan-Einot-Gabriel-Welsh (REGW) stepwise multiple comparison procedure was used to compare means because of its power and control of the experimentwise type I error rate (Day and Quinn, 1989). Captures of male beetles were similar to those of female beetles in all experiments. In experiments 1 and 5 data for the two sexes were pooled to compensate for low power due to relatively few replicates and low trap catches. In all cases $\alpha = 0.05$.

RESULTS

In experiment 1, spruce beetles were significantly more attracted to spruce beetle lures than to unbaited controls or traps baited with the pheromone of *D. affaber* (Figure 1). Attraction of spruce beetles to spruce beetle lures plus the pheromone of *D. affaber* was intermediate between that to unbaited controls

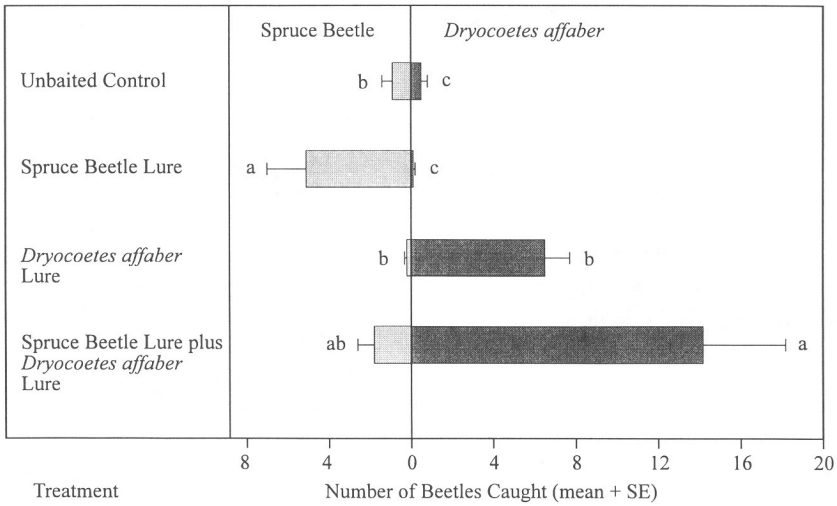

FIG. 1. Numbers of spruce beetles and *Dryocoetes affaber* captured in multiple funnel traps in experiment 1 (May 28–July 7, 1993) Slate Creek, Princeton, British Columbia. Spruce beetle lures consisted of frontalin released at 2 mg/24 hr and α-pinene released at 50–80 mg/24 hr. *Dryocoetes affaber* pheromone consisted of a 1:1 mixture of (±)-*exo*- and (+)-*endo*-brevicomin released at 0.2 mg/24 hr. $N = 10$. Bars for each species with the same letter are not significantly different, REGW test $P < 0.05$.

and spruce beetle lures alone. *Dryocoetes affaber* was significantly attracted to its own pheromone but not to spruce beetle lures (Figure 1). Attraction to its own pheromone was significantly enhanced in the presence of the spruce beetle lure.

In experiments 2–4 the numbers of spruce beetles captured were significantly different between the two collection periods. Because high numbers of beetles were caught early in the flight period and low numbers later on at the time of the second collection, the overall means were intermediate with large overall variances. Nevertheless, differences in responses to different treatments were similar between the two time periods and were significant when blocking factors for time and replicate were included.

In experiment 2 spruce beetles of both sexes were attracted in significant numbers to traps baited with spruce beetle lures alone or combined with (−)- or (±)-*exo*-brevicomin (Figure 2). The presence of (+)-*exo*-brevicomin reduced catches slightly, but significantly to a level not different from that to unbaited control traps (Figure 2). Catches of *D. affaber* were very low and were not significantly different between treatments.

Attraction of spruce beetles in experiment 3 was similarly reduced by 42%

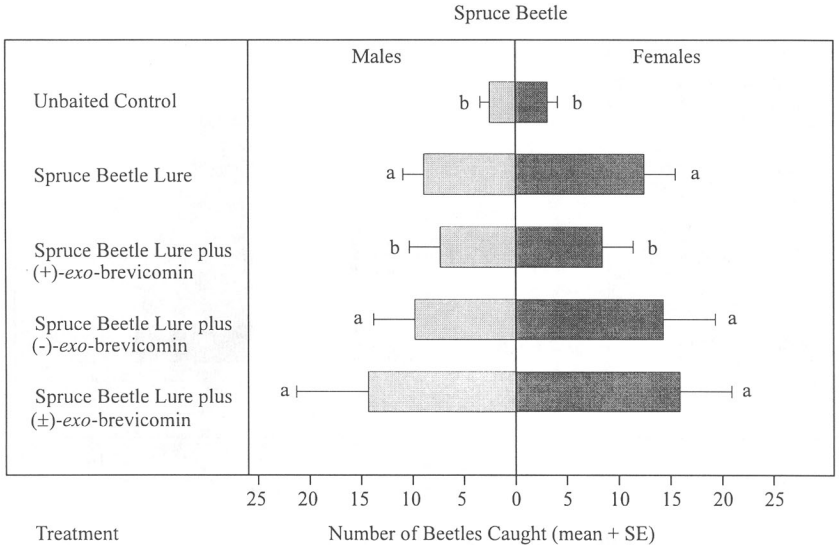

FIG. 2. Numbers of male and female spruce beetles captured in multiple funnel traps in experiment 2 (April 26–June 29, 1994) in Arastra Creek, Princeton, British Columbia. Spruce beetle lures consisted of frontalin released at 2 mg/24 hr and α-pinene released at 50–80 mg/24 hr. Enantiomers of *exo*-brevicomin were released at 0.2 mg/24 hr. $N = 20$. Bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$.

when spruce beetle lures were combined with (+)- or (±)-*endo*-brevicomin (Figure 3). *Dryocoetes affaber* was attracted to spruce beetle lures combined with either (+)- or (±)-*endo*-brevicomin.

In experiment 4 spruce beetles were attracted at significant levels only to traps baited with spruce beetle lures (Figure 4). Addition of (±)-*exo*-brevicomin, (±)-*endo*-brevicomin, or both to spruce beetle lures reduced the numbers of spruce beetles captured by 75%, 87%, and 77% respectively, levels not significantly different from that to unbaited control traps (Figure 4). *Dryocoetes affaber* was significantly attracted to spruce beetle lures plus (±)-*endo*-brevicomin alone or combined with (±)-*exo*-brevicomin but not to spruce beetle lures plus (±)-*exo*-brevicomin alone.

The results of experiment 5 were very similar to those of experiment 1. The attraction of spruce beetles to the combination of spruce beetle and *I. tridens* attractants was reduced to a level intermediate between that to unbaited controls and spruce beetle lures (Figure 5). *Ips tridens* was significantly attracted to its

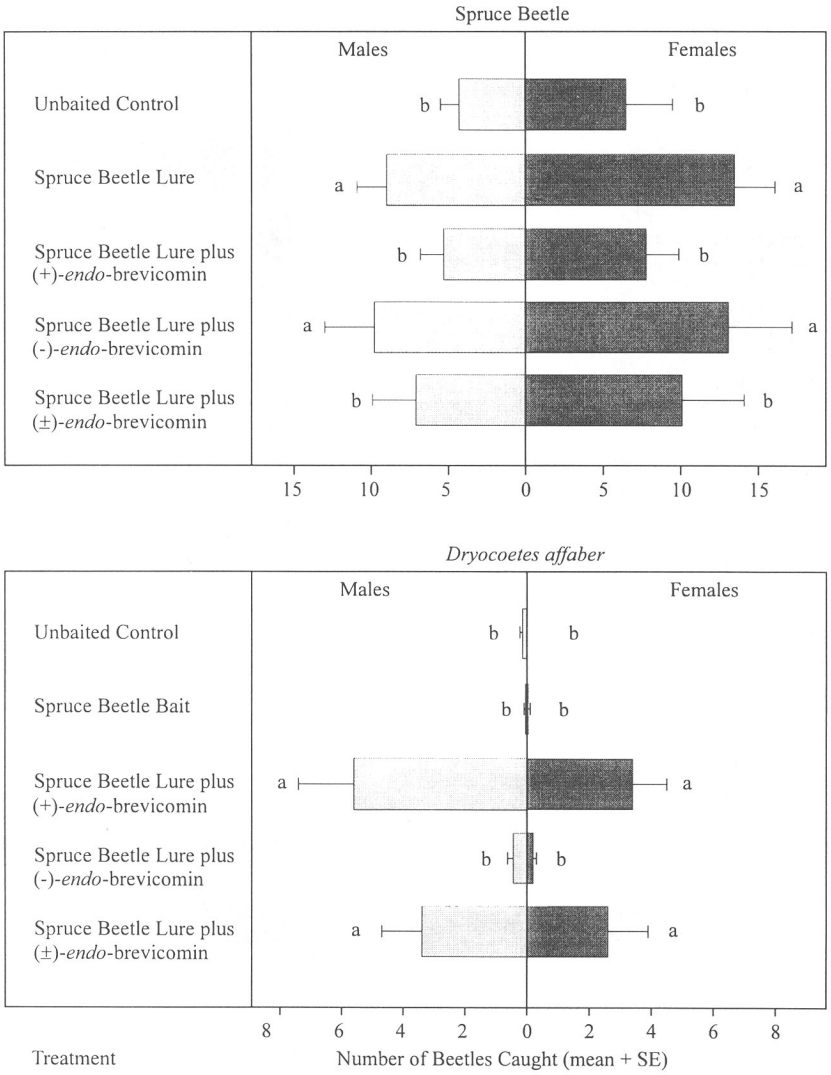

FIG. 3. Numbers of male and female spruce beetles and *Dryocoetes affaber* captured in multiple funnel traps in experiment 3 (April 26–June 29, 1994) in Arastra Creek, Princeton, BC. Spruce beetle lures consisted of frontalin released at 2 mg/24 hr and α-pinene released at 50–80 mg/24 hr. Enantiomers of *endo*-brevicomin were released at 0.2 mg/24 hr. $N = 20$. Bars with the same letter within each species and sex are not significantly different, REGW test, $P < 0.05$.

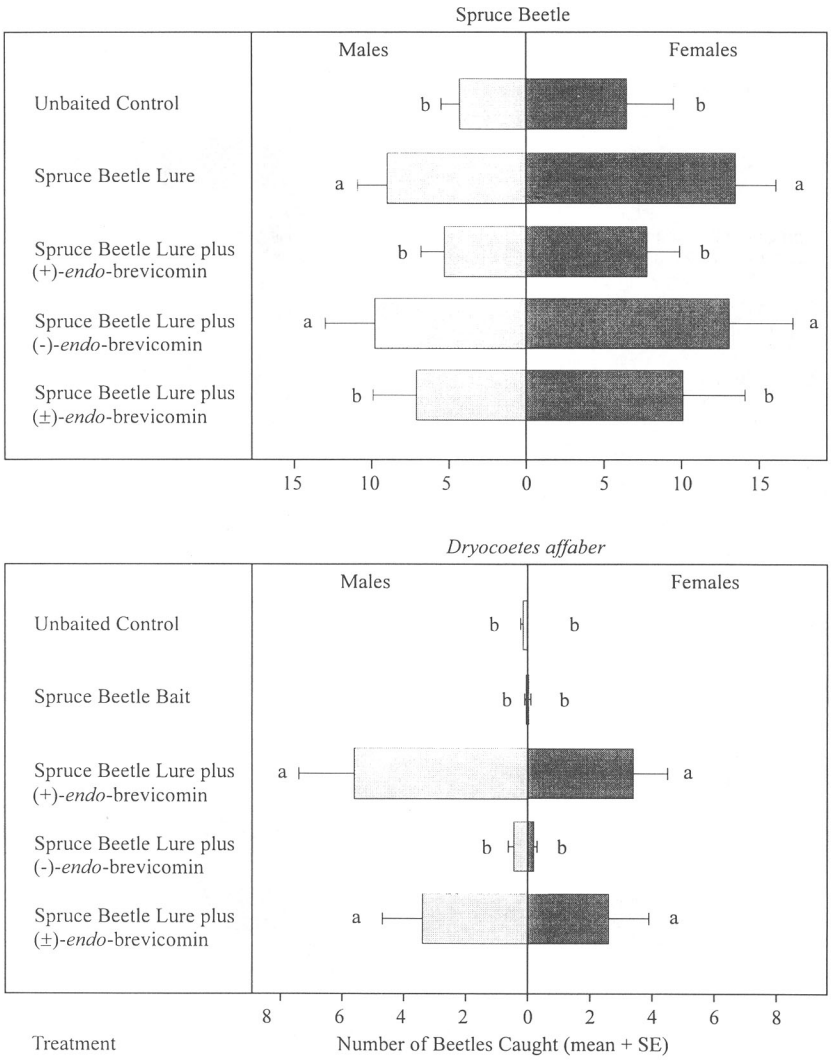

FIG. 4. Numbers of male and female spruce beetles and *Dryocoetes affaber* captured in multiple funnel traps in experiment 4 (June 29–August 17, 1994) in Arastra Creek, Princeton, British Columbia. Spruce beetle lures consisted of frontalin released at 2 mg/24 hr and α-pinene released at 50–80 mg/24 hr. The (±)-*exo*- and (±)-*endo*-brevicomin were released at 0.2 mg/24 hr. $N = 20$. Bars with the same letter within each species and sex are not significantly different, REGW test, $P < 0.05$.

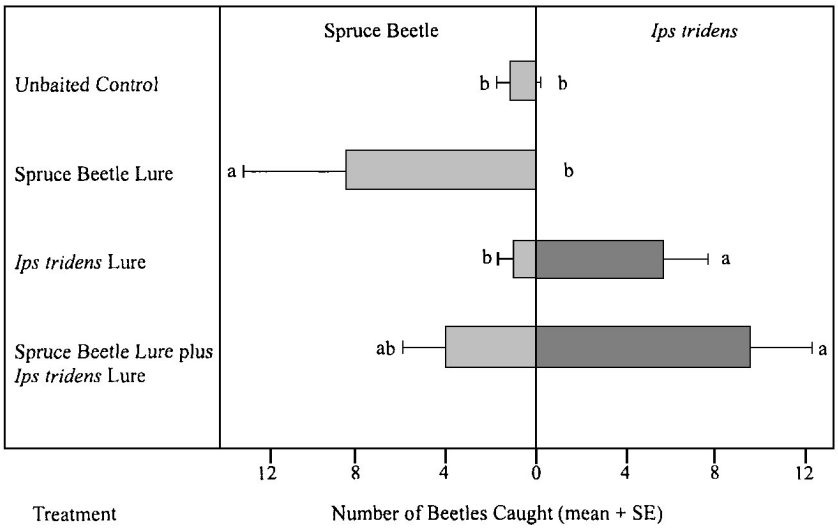

FIG. 5. Numbers of spruce beetles and *Ips tridens* captured in multiple funnel traps in experiment 5 (May 28–July 7, 1993) Slate Creek, Princeton, British Columbia. Spruce beetle lures consisted of frontalin released at 2 mg/24 hr and α-pinene released at 50–80 mg/24 hr. *Ips tridens* lures consisted of (±)-ipsdienol and (−)-*cis*-verbenol released at 0.6 and 1.8 mg/24 hr, respectively. $N = 10$. Bars for each species with the same letter are not significantly different, REGW test $P < 0.05$.

putative pheromone alone or in combination with spruce beetle lures, but unlike *D. affaber* its attraction was not significantly enhanced in the presence of the spruce beetle lure (Figure 5).

In experiment 6 spruce beetles of both sexes were significantly more attracted to traps baited with spruce beetle lures than to unbaited controls. The presence of (+)- or (±)-ipsdienol significantly reduced spruce beetle attraction by 85% to a level that did not differ from that to unbaited control traps (Figure 6).

DISCUSSION

The experimental results uphold all three hypotheses, at least in part. Attraction of spruce beetles to traps baited with the aggregation pheromone frontalin plus the host kairomone α-pinene was reduced by up to 87% by both pheromones of *D. affaber* (Figures 1 and 4). Spruce beetle attraction was reduced by the naturally produced (+) enantiomers of both *exo*- and *endo*-brevicomin

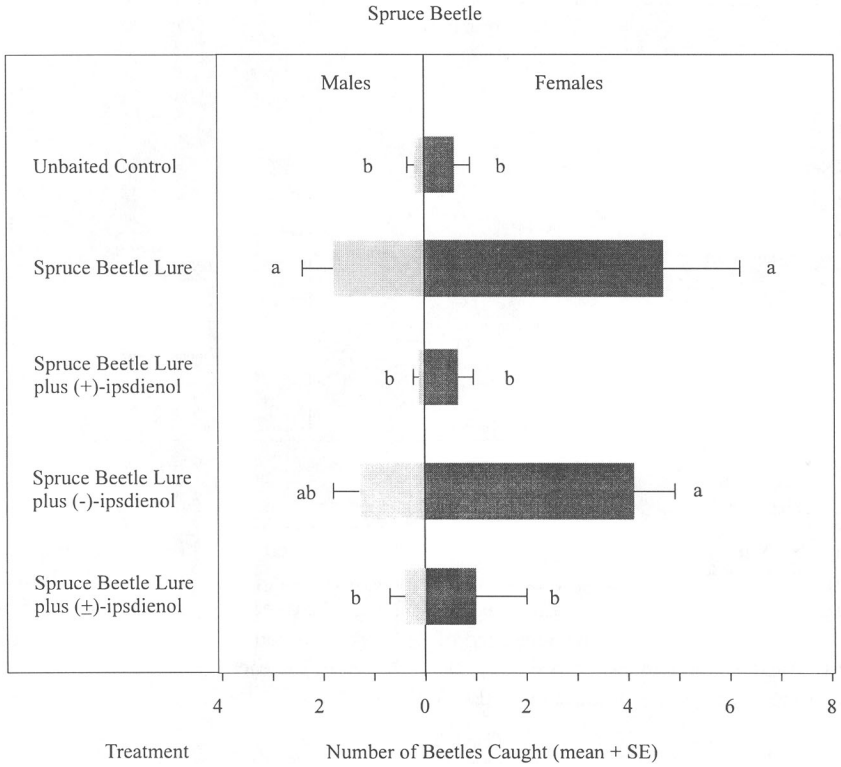

FIG. 6. Mean numbers of male and female spruce beetles captured in multiple funnel traps in experiment 6 (July 14–August 17, 1994) in Arastra Creek, Princeton, British Columbia. Spruce beetle lures consisted of frontalin released at 24 mg/24 hr and α-pinene released at 50–80 mg/24 hr. Enantiomers of ipsdienol were released at 0.2 mg/24 hr. $N = 20$. Bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$.

(Figures 2 and 3). (±)-*endo*-Brevicomin also reduced attractiveness of the spruce beetle lure in experiments 3 and 4 (Figures 3 and 4) and (±)-*exo*-brevicomin in experiment 4 (Figure 4). (±)-Ipsdienol as well as its (+) enantiomer reduced attraction of the spruce beetle to the spruce beetle lure (Figure 6). On the other hand, spruce beetle lures enhanced attraction of *D. affaber* to its pheromone (Figure 1), but no such effect was observed for *I. tridens* (Figure 5).

Several species of bark beetles are known to produce and respond to enantiospecific pheromones (Birch, 1984; Borden, 1985; Byers, 1989). Specificity in the enantiomeric composition of pheromones may be an important mechanism in maintaining breeding isolation between sympatric congeners. The western

balsam bark beetle, *Dryocoetes confusus* Swaine, is sympatric with *D. affaber* and shares the aggregation pheromones (+)-*exo*- and (+)-*endo*-brevicomin. Optimal attraction of *D. affaber* is elicited by a 1:2 ratio of the two compounds, whereas a 9:1 ratio results in optimal attraction of *D. confusus* (Camacho et al., 1993). Attraction of *D. affabar* was reduced when (+)-*exo*-brevicomin was released with (+)-*endo*-brevicomin at the 9:1 ratio and (−)-*endo*-brevicomin was also found to be inhibitory (Camacho et al., 1994). (+)-*endo*-Brevicomin was inhibitory to *D. confusus* when released with (+)-*exo*-brevicomin at a 1:1 ratio (Camacho et al., 1993; Stock et al., 1994) and (−)-*exo*-brevicomin disrupted attaction to the optimal blend (Camacho et al., 1993).

Olfactory receptor cells specific to enantiomers of aggregation pheromones are present in *I. pini* and *I. paraconfusus* (Mustaparta et al., 1980, 1984), *I. typographus* (Tømmeros et al., 1984), and *Scolytus scolytus* (F.) (Wadhams et al., 1982). Perception of the species-specific enantiomeric blends of (+)-*exo*- and (+)-*endo*-brevicomin by *D. affaber* and *D. confusus* is likely similarly achieved by specific olfactory receptor cells. Because attraction of spruce beetles is reduced by naturally produced (+) enantiomers of *exo*- and *endo*-brevicomin (Figures 3 and 4), they may also possess enantiospecific receptor cells. This suggests that spruce beetles and *D. affaber* coevolved since it would be adaptive for spruce beetles to recognize the enantiospecific pheromones of competing secondary species.

Spruce beetle attraction was also reduced by (±)- and (+)-ipsdienol. We have found that *I. tridens* produces ipsdienol in a 1:4 ratio of (+)- and (−)-enantiomers (unpublished). Ipsdienol is produced and utilized by many scolytid species (Borden, 1982). The (+) enantiomer may be a major pheromone component for other secondary species associated with the spruce beetle. Production of some (+)-ipsdienol by *I. tridens* would be adaptive since it reduces spruce beetle attraction and would minimize competitive interactions.

Combinations of pheromones of several associated secondary species may further reduce spruce beetle attraction. Additional research is needed to test the effect of combining the pheromones of *I. tridens*, *D. affaber*, *I. perturbatus*, *P. rufipennis*, and other secondary scolytids on spruce beetle attraction.

Recognition of the pheromones of sympatric species and avoidance of already colonized resources would be adaptive whenever interspecific encounters could result in reduced fitness (Borden, 1996). Weakened or dead host trees colonized by secondary species would generally be unsuitable for primary bark beetles that attack freshly fallen or living trees. Therefore, it would be adaptive for primary bark beetles to recognize and avoid secondary species. On the other hand, secondary species attack only downed trees or those already weakened or killed by primary bark beetles. Therefore, secondary species may exploit the pheromones of tree killing bark beetles to aid in host location.

Interspecific pheromonal inhibition is also common among lepidopterans.

For instance, the bollworm, *Heliothis zea* Boddie, and the tobacco budworm, *Heliothis virescens* F., share four pheromone components, (*Z*)-11-, (*Z*)-9-, and (*Z*)-7-hexadecenal and hexadecanal. Three additional components, (*Z*)-9-tetradecenal, tetradecanal, and (*Z*)-11-hexadecen-1-ol, in *H. virescens* are responsible for pheromone specificity and reproductive isolation of the two species (Stadelbacher et al., 1983).

Our results indicate that spruce beetles recognize the pheromones of secondary species and thereby probably are able to avoid direct competitive interactions. They suggest that this phenomenon could be exploited by using the pheromones of secondary species, or less expensive substitutes to induce competitive displacement or exclusion of the spruce beetle by secondary species. Such treatments could reduce or replace the use of arsenical-treated lethal trap trees (Hodgkinson, 1985). Baiting susceptible hosts for secondary species would have two positive effects: (1) inducing attack by the secondary species, and (2) partially repelling spruce beetles. The efficacy of competitive exclusion in reducing spruce beetle attack density, gallery lengths per square meter, and progeny density has been experimentally demonstrated in felled trap trees baited with semiochemical attractants for either or both of the secondary species (Poland, 1997). These tactics may provide a technique for spruce beetle management, especially in remote or environmentally sensitive areas.

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INFLUENCE OF LIGHT AND PHOTOSYNTHESIS ON ALKALOID CONCENTRATION IN LARKSPUR

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Abstract—Concentrations of toxic norditerpenoid alkaloids vary greatly in tall larkspur (*Delphinium barbeyi*) and may be influenced by environmental stress. We evaluated the effect of shade, darkness, and inhibition of photosynthesis on toxic alkaloid concentration. In plants treated with metribuzin to inhibit photosynthesis, alkaloid concentration increased, but dry weight of the plants decreased as growth ceased, leaving absolute alkaloid content similar to that of control plants. Short-term shade (70% reduction in sunlight for three days), dark treatments from leaves collected at night, and aluminum foil covered leaves all increased alkaloid concentration in comparison to untreated control plants. It appears that absolute amounts of alkaloids remained the same, but the mass of stressed plants declined as nonstructural carbohydrates were depleted, thus increasing the relative concentration of alkaloids. We conclude that norditerpenoid alkaloids in larkspur do not respond to short-term light stress. Alkaloid concentration was lower in larkspur plants growing beneath forest canopy and in potted plants in a long-term shade study (70% reduction in sun light for 21 days) than plants growing in open sunlight. Long-term shade may have reduced synthesis of norditerpenoid alkaloids, particularly in the earlier developmental stages of the plant. Shade stress or photosynthesis inhibition apparently did not increase norditerpenoid alkaloid synthesis, which contrasts with the carbon/nutrient balance theory of plant defense.

Key Words—*Delphinium barbeyi*, norditerpenoid alkaloids, methyllycaconitine, 14-deacetylnudicauline, environmental stress, shade, diurnal, dark, photosynthesis inhibition, metribuzin, carbon/nutrient balance theory.

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INTRODUCTION

Larkspur species (*Delphinium* spp.) contain norditerpenoid alkaloids that are acutely toxic to cattle (Olsen 1978). Larkspurs are also palatable (Pfister et al., 1988), thus causing severe poisoning problems on mountain rangeland (Nielsen and Ralphs 1988). Concentrations of toxic alkaloids are known to vary between species, locations, plants within a population, plant part, and phenological growth stage (Manners et al., 1993; Pfister et al., 1994; Ralphs et al., 1988, 1997). The reason why the toxin level varies is not known.

The resource availability theory of plant defense suggests that availability of nutrients in the environment is the major factor influencing the type and amount of defense compounds (Coley et al., 1985). This theory predicts that rapidly growing plants in resource-rich habitats contain low levels of highly mobile toxins, such as alkaloids and cyanogenic glycosides. These plants exhibit biochemical and morphological plasticity to allow them to take advantage of pulses in resource availability. Nitrogen (N) is taken up early in the growing season in excess of the plant's needs for growth. Excess N is available to be synthesized into N-based qualitative or toxic defense compounds that can be rapidly induced upon injury to protect the plant from further herbivory (Mooney et al., 1983).

The carbon/nutrient balance theory further explains changes in concentrations in defense compounds (Bryant et al., 1983, 1992). If light becomes limiting (i.e., shade or cloudy weather) to plants growing in nutrient-rich environments, the decline in photosynthesis may limit carbohydrates for growth and carbon-based defenses, but nutrient uptake continues, leaving excess N that could be shunted to N-based defense compounds such as alkaloids.

The objective of this study was to determine the influence of light and photosynthesis on norditerpenoid alkaloid concentration in tall larkspur (*Delphinium barbeyi*). The carbon/nutrient balance theory predicts that shade stress or photosynthesis inhibition should increase alkaloid concentration.

METHODS AND MATERIALS

Larkspur Alkaloids

The toxic compounds in larkspurs have been identified as norditerpenoid alkaloids (Figure 1). Alkaloids that contain the *N*-(methylsuccinimido) anthranilic ester group (referred to as MSAL alkaloids) are the most toxic (Manners et al., 1995), with methyllycaconitine (MLA) and 14-deacetylnudicauline (DAN) being the two most prominent toxic alkaloids in tall larkspur. The alkaloids MLA and DAN were extracted in ethanol and chloroform, then quantified by normal-phase, isocratic high-pressure liquid chromatography (HPLC) (Manners

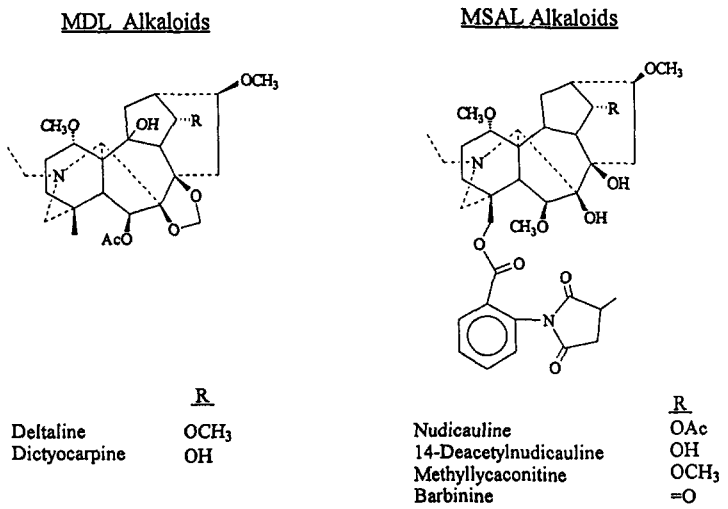

FIG. 1. Nornorditerpenoid alkaloids in larkspur species. The two major classes of alkaloids are methylenedioxylcoctonine (MDL) and *N*-(methylsuccinimido)anthranoyllycoctonine (MSAL) alkaloids.

and Pfisters, 1983) in the shade and dark stress studies. These alkaloids are of similar toxicity (Manners et al., 1995); therefore, they were summed to express toxic alkaloid concentration. In the study using metribuzin to inhibit photosynthesis, the MSAL fraction and total norditerpenoid alkaloid concentration [which includes MSAL and methylenedioxylycoctonine (MDL) type alkaloids] were measured by Fourier transform infrared spectroscopy (FTIR) (Gardner et al., 1997). They were extracted in chloroform and 1% H_2SO_4, and IR spectra were collected using a Nicolet Magna 550 FT-IR spectrometer (Nicolet Instrument Corp., Madison, Wisconsin). This new method used HPLC values from previous studies to calibrate the predictive equation. The alkaloids MLA and DAN comprise most of the MSAL fraction, therefore the MSAL fraction is also referred to as toxic alkaloids in the metribuzin study. Samples from the greenhouse studies were frozen and freeze-dried prior to extraction. Samples from the field were dried in a forced air oven at 60°C for 48 hr. A comparison was made between drying methods, and there were no differences in either toxic or total alkaloids.

Long-Term Shade Stress

Field Studies, 1992. Samples of tall larkspur and duncecap larkspur (*D. occidentale*) were collected in conjunction with a large sampling program to

assess the toxicity of larkspur species and populations (Ralphs et al., 1997). Leaves from larkspur plans growing under conifer or aspen canopy, and adjacent plants growing in open sunlight were harvested at two-week intervals during the growing season. Tall larkspur samples were collected in a subalpine fir community 24 km east of Manti, Utah, at an elevation of 3000 m, and in an aspen community 46 km east of Salina, Utah, at an elevation of 3100 m. Duncecap larkspur samples were collected in aspen communities 32 km west of Oakley, Idaho, at 2500 m, and 28 km east of Jackpot, Nevada, at 2300 m. Samples were harvested around noon to reduce any effects of dirunal variation in alkaloid concentration. Five leaves from 30–50 plants growing in the sun, and five leaves from a similar number of plants growing in shade were harvested and composited into single samples (sun or shade treatments) for each time at each location. The samples were analyzed for toxic alkaloids by HPLC. The toxic alkaloid concentrations in shade and sun plants were compared by paired *t* tests.

Potted Plants, 1993. Tall larkspur plants were established from seed collected from the Manti, Utah, site and were grown in the greenhouse. After overwintering in a dark cold room (5°C), 30 plants were taken out on March 26, 1993, and cloned by splitting each plant in half. The roots were rinsed in a dilute Clorox solution, then dipped in a root-stimulating hormone solution. They were planted in a growing medium of equal parts of soil, periolite, and peat in 20-cm pots and allowed to grow in the greenhouse. These plants were fertilized biweekly with Peters 20-20-20 NPK garden fertilizer. In addition to producing clones, the newly potted plants prevented any suppression of alkaloid synthesis that may be inherent in root-bound potted plants (Baldwin, 1988). On May 10, 10 cloned pairs were placed outside to acclimate to outdoor growing conditions for three weeks. One of each cloned pair was then randomly selected and placed under a shade cloth canopy designed to filter out 70% of light. The other plant was left in the open sun. The plants were left in these treatments for three weeks. Midday photosynthetic photon flux (PPF) was measured by a Li-Cor LI-185B photometer and averaged 2000 μmol/m^2/sec in the open and 750 μmol/m^2/sec in the shade. At the end of three weeks, the leaves were harvested, frozen, freeze-dried, and ground to pass through a 1-mm screen in a Wiley mill. Alkaloids were extracted and individual toxic alkaloids were quantified by HPLC. Differences in toxic alkaloids between treatments were determined by paired *t* tests.

Short-Term Shade Stress

Potted Plants, 1994. Cloned plants were overwintered in a dark cold room, then taken out and allowed to grow in the greenhouse during the spring. The foliage was clipped back to 2.5 cm from the soil surface, and 25 pairs of clones were placed outside to regrow and acclimate to the outside environment on May

13. They were allowed to grow for 25 days before being placed in the respective shade treatments for three days. As before, one of each clone was randomly selected and placed under 70% shade cloth, while the other was left in the open sun. Mean irradiation levels at mid day were 2067 μmol/m^2/sec in the open sun and 173 μmol/m^2/sec in the shade, and temperatures were 31°C and 25°C, respectively. Leaves were harvested from the plants at midday on the third day, frozen, freeze-dried, ground, and analyzed by HPLC.

Potted Plants, 1995. Cloned plants were overwintered in the cold room, then taken out in March and grown in the greenhouse. On June 21, 20 cloned pairs were clipped near the soil surface and then allowed to grow outside in open sunlight. One of each cloned pair was placed in the 70% shade treatment on July 26, then the leaves from both plants of the clone were harvested three days later, frozen, freeze-dried, and toxic alkaloids were measured by HPLC. Toxic alkaloids in shade and sun plants were compared by paired *t* tests.

Dark Stress on Field Plants, Salina 1994

Diurnal. The study site was located near the 1992 Saline study site at 3000 m elevation. Larkspur plants were in the bud elongation stage of development in mid-July. Ten larkspur plants were selected; 10 stems from each plant were harvested at 05:00 hr (before dawn) for the dark treatment, and another 10 stems from the same plant were harvested at noon for the sun treatment. Leaves from the top two thirds of the stalks were plucked and placed in a separate paper bag for each plant. The samples were returned to the laboratory, dried in a forced-air oven, and then extracted and analyzed for toxic alkaloids by HPLC. The alkaloid concentration was analyzed by paired *t* tests comparing samples from the same plant during dark and sun periods.

Aluminum Foil on Leaves. The study site was near the diurnal study at Salina at 3200 m elevation. It was on a snowdrift site, and the larkspur plants were at a younger stage of development (late vegetative). Five plants were selected on each of four days (July 15, 16, 20, 21, 1994). Aluminum foil was placed on alternate leaves in the early afternoon, then all the leaves were harvested late the next afternoon. Covered leaves and leaves exposed to sunlight from each plant were placed in separate bags, dried in a forced-air oven, and analyzed by HPLC. Data were analyzed by paired *t* tests.

Metribuzin Treatment to Inhibit Photosynthesis

1994 Study. The herbicide metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one] inhibits photo system II in the photosynthetic process (Devin et al., 1993). It not only stops photosynthesis, but toxic oxygen species are created, which in turn form caustic hydroxyl radicals that

rapidly disrupt cell membranes. Whereas the shade stress and dark treatments reduced or stopped photosynthesis and subsequent carbohydrate synthesis, metribuzin rapidly depletes energy pools and disrupts membranes of the chloroplast, thus stopping any biosynthetic pathways in the chloroplast. If alkaloid synthesis occurs in the chloroplast, we would expect this treatment to reduce alkaloid concentrations.

The site for the study was 50 km east of Salina, Utah, at 2700 m elevation. Four larkspur patches were split in half, and one half was randomly selected and sprayed with metribuzin at a rate of 140 g ai (active ingredient)/ha (2 oz/ac). A backpack sprayer was used with a 4-nozzle boom, putting out 173 liters of total spray solution per hectar. Five leaves were harvested from 30–50 plants in each plot on day 0 before spraying, and 1, 3, 7, and 14 days after treatment. The samples were oven dried and toxic and total alkaloids were estimated by Fourier transform infrared spectroscopy (FTIR). The concentrations of toxic and total alkaloids were compared by analysis of variance (ANOVA) in a split-plot design, with the treatments as the main plot and time the split plot.

1995 Study. The study site was in the next drainage valley 2 km west of the 1994 site in Salina Canyon. Forty larkspur plants were staked, and half were sprayed with metribuzin at the same herbicide concentration as used in the 1994 trial. Each plant was sprayed to wetness using a single solid cone nozzle. A single stalk from each plant was harvested on day 0 before treatment, then 1, 4, 7, and 14 days after treatment. The stalk was weighed when harvested and again after drying in a forced-air oven, and dry weight and percentage dry matter were calculated. The entire stalk, including stem, leaves, and head was ground and analyzed for alkaloids in order to determine the alkaloid concentration and absolute amount of alkaloid in the stalk. Toxic and total alkaloids were estimated by FTIR. Toxic and total alkaloid concentration was multiplied by the dry weight of each stalk to obtain absolute amount of alkaloids in each stalk. The alkaloid concentrations and absolute amounts of alkaloids were compared between the metribuzin-treated plants and untreated control plants in a repeated measures ANOVA over time.

Variability Among Plants

The variability in toxic alkaloid concentration between individual plants was partitioned by analysis of variance (ANOVA) for the 1994 field diurnal and aluminum foil studies and in the 1995 metribuzin study to inhibit photosynthesis. The data for the diurnal and aluminum foil study were arranged in a simple randomized block design with plants serving as blocks. In the metribuzin study, the data were arranged in a randomized-block, split-plot design with individual plants as blocks and days as the split plot. The proportions of variation due to treatment, plant, and day (in the metribuzin study), were calculated by the ratio

of sum of squares for treatment, plant, and day to the total sum of squares (Coleman et al., 1987).

RESULTS

Alkaloid Profile

There was a marked difference in the amount and proportion of individual alkaloids between larkspur plants in pots started in the greenhouse and plants growing naturally in the field. Toxic alkaloids in potted plants in the 1993 and 1995 shade-stress studies were an order of magnitude lower than in field-grown plants in the 1992 and 1994 studies (Table 1). The concentraitons in potted plants were slightly higher in the 1994 shade study, but still substantially lower than in the field grown plants. The difference in toxic alkaloid concentration between field and potted plants is not likely due to the root-bound condition of the plants (Baldwin, 1988). The 1993 plants were newly split and had adequate room for growing roots. In a related study, newly cloned plants were actually lower in toxic alkaloids than plants growing in the same pot for three years (Ralphs, unpublished data). It is unlikely that larkspur alkaloids are produced in root tips and translocated up through the xylem to the foliar parts, as suggested by Baldwin (1991) in wild tobacco (*Nicotiana sylvestris*).

The composition of alkaloids also differed between potted plants and plants growing in the field. The concentration of DAN was similar in potted plants and those growing in the field (0.2–0.5 mg/g), but MLA increased from 0.2–0.9 mg/g in the potted plants to 5–10 mg/g in the field plants. The difference in toxic alkaloids between potted plants and those growing in the field was due almost entirely to the increase in MLA.

In the metribuzin studies, alkaloids were quantified by infrared spectroscopy (FTIR), which gives the toxic alkaloid fraction and total norditerpenoid alkaloid concentration. The toxic alkaloids comprised 40% of total norditerpenoid alkaloids at the two Salina sites.

Long-Term Shade Stress

Leaves of tall and duncecap larkspur growing in open sunlight in the 1992 field study contained higher concentrations of toxic alkaloids than plants growing in the shade of either aspen or conifer ($P < 0.07$, Table 1). We speculate that reduction in light under tree canopy reduced synthesis of toxic alkaloids in the leaves. Other environmental differences between the shaded and open sites (temperature, soil type, fertility and moisture) may have also contributed to the difference in toxic alkaloids.

In the 1993 study, cloned larkspur plants grown in the open sun also had

TABLE 1. INFLUENCE OF SHADE AND DARK STRESS ON TOXIC ALKALOID CONCENTRATION IN LARKSPUR PLANTS

Study	Species/location	Year	*N*	Conc. (mg/g)		*P*
				Sun	Shade or dark	
Long-term shade						
Field	Tall	1992	9	4.9 ± 1.3	4.0 ± 1.0	0.02
	Duncecap	1992	8	3.1 ± 0.7	2.3 ± 0.4	0.07
21-day shade	Potted plants	1993	8	0.4 ± 0.07	0.24 ± 0.0	0.01
Short-term shade						
3-day shade	Potted plants	1994	24	1.1 ± 0.65	1.5 ± 0.91	0.05
	Potted plants	1995	19	0.24 ± 0.0	0.33 ± 0.0	0.06
Dark stress						
Diurnal	Salina	1994	10	5.7 ± 0.8	6.4 ± 0.97	0.08
Aluminum foil	Salina	1994	20	5.3 ± 0.6	5.9 ± 0.7	0.02

a higher concentration of toxic alkaloids than those grown in shade for three weeks ($P = 0.01$, Table 1). Plants in the shade were developmentally younger, which should have made them higher in alkaloid concentration (Ralphs et al., 1997). This suggests that there may have been a real decline in toxic alkaloid concentration in long-term shade stressed plants. Majak et al. (1977) also reported that miserotoxin (3-nitropropanol) in timber milkvetch (*Astragalus miser* var. *serotinus*) was higher in plants growing in open grassland compared to plants growing under forest canopy.

Short-Term Shade and Dark Stress

Plants stressed for short periods of time in shade or darkness increased toxic alkaloid concentration. In the three-day shade studies in 1994 and 1995, shade stressed plants had higher toxic alkaloid concentrations than the plants growing in open sun ($P < 0.06$, Table 1). In the 1994 diurnal field study at Salina, plants harvested before dawn were higher in toxic alkaloid concentration than those harvested at noon although not by a statistically significant amount ($P = 0.08$, Table 1). Leaves in total darkness in the aluminum foil study also had higher mean toxic alkaloid concentration than those exposed to sunlight ($P = 0.02$, Table 1).

The slight increase in alkaloid concentration in these four studies could have been due to the relative decrease in biomass of the plants under stress. Unrestricted photosynthesis in plants exposed to full sunlight would continue to produce carbohydrates and other metabolites, thus increasing plant biomass and reducing the relative concentration of a constant amount of alkaloids. Restricted photosynthesis in shade and dark stressed plants may have reduced the relative biomass as carbohydrates were depleted or translocated out of the leaves, thus increasing the concentration of alkaloids. Smart et al. (1994) reported that total nonstructural carbohydrates (TNC) in wheat declined 50% from peak concentration during the day, to a low concentration at night. This accounted for a 15% reduction in dry weight of the shoot. Furthermore, the influence of shade within the wheat canopy (80% reduction in PPF from top to bottom) also reduced TNC 50% in bottom leaves compared to top leaves, resulting in a decrease of 5% in the dry weight of the leaves. The concentration of toxic alkaloids in our study increased an average of 11% in the dark treatments of the diurnal and aluminum foil field studies, and 39% in the shade studies using potted plants. If TNC depletion in larkspur leaves in dark and shade were similar to wheat, this reduction in mass could account for the relative increase in concentration of toxic alkaloids.

Metribuzin Treatment

Toxic and total alkaloid concentrations increased in plants treated with metribuzin over time, compared to a slight reduction in concentration in control

plants in both years ($P < 0.02$, Figure 2). However, there was no difference in absolute amount of toxic alkaloids in larkspur stalks between treatments ($P = 0.48$, Figure 3A). The absolute amount of alkaloid was calculated by multiplying the dry weight of the stalk (Figure 3C) by its toxic alkaloid concentration (Figure 3B). The toxic alkaloid concentration in control plants gradually declined over time (Figure 3B). This is the typical trend over the growing season (Man-

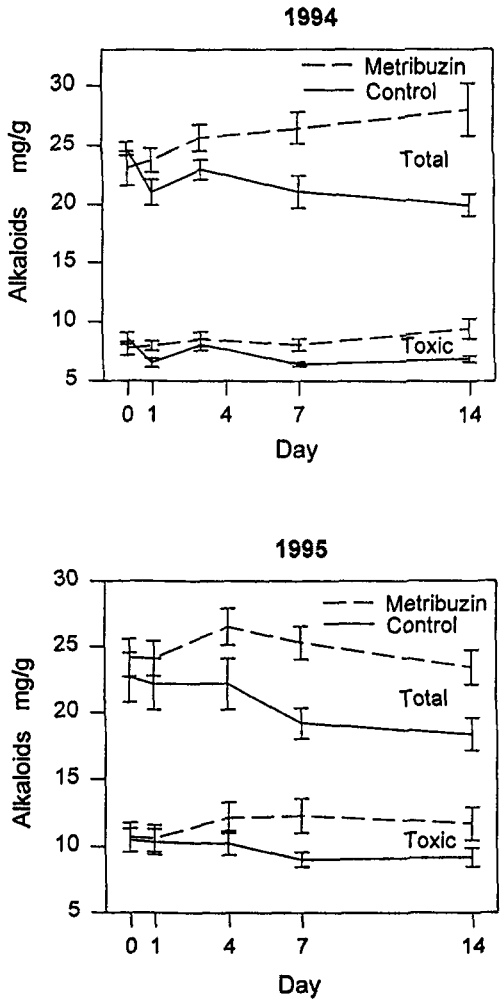

FIG. 2. Toxic and total alkaloid concentration in larkspur plants treated with metribuzin in the 1994 and 1995. Error bars are standard errors of the means.

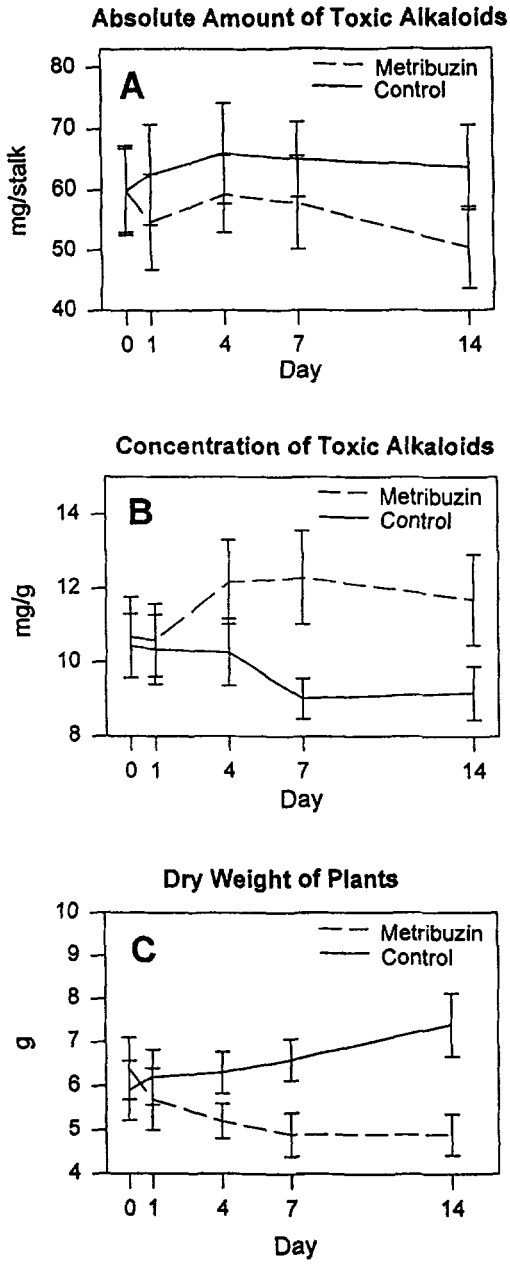

FIG. 3. (A) Absolute amount of toxic alkaloids, (B) concentration of toxic alkaloids, and (C) dry weight of larkspur stalks treated with metribuzin to inhibit photosynthesis, and untreated control plants. Error bars are standard errors of the means.

ners et al., 1993; Pfister et al., 1994; Ralphs et al., 1997). However, the dry weight of control plants increased as the plants continued to grow (Figure 3C), causing the absolute amount of toxic alkaloids to remain relatively constant (Figure 3A). In plants treated with metribuzin, toxic alkaloid concentration increased (Figure 3B), but dry weight of the stalk decreased (Figure 3C) as photosynthesis ceased and carbohydrate synthesis stopped. The plants did not desiccate; the percentage dry matter remained $<20\%$ and was similar to control plants. Thus, the absolute amount of alkaloid in metribuzin-treated plants remained similar to the controls, even though their alkaloid concentrations and dry weights changed in opposite directions. Total alkaloids responded in a similar manner (data not shown).

Variation Among Plants

We investigated the variation in toxic alkaloid concentration among individual plants by partitioning the ratio of sum of squares in the ANOVA. The difference in toxic alkaloid concentration among plants was much greater than the difference between treatments. In the 1994 diurnal and aluminum foil studies comparing samples from the same plant, differences between plants accounted for 95% and 96% of the variability in the statistical model, while treatment differences accounted for only 1%. Toxic alkaloid concentration among the plants ranged from 1 to 12 mg/g, compared to the average of 5 and 6 mg/g for dark and light treatments, respectively. In the 1995 metribuzin study, treatment accounted for 5% of variation, day (change over two weeks) accounted for 4%, but plant differences accounted for 51% of the variation. Toxic alkaloid concentrations ranged from 1 to 23 mg/g, compared with the treatment means of 9 and 11 mg/g for the control and photosynthesis-inhibited treatments, respectively. Manners and Pfister (1996) also reported large variation in alkaloid concentration between larkspur plants. Other studies reported great variation in toxin concentration among plants in a population (Vrieling et al., 1993; Van Dam and Vrieling, 1994; Zangerl and Berenhaum, 1990; Coleman et al., 1987).

DISCUSSION

There was no difference in absolute amount of toxic and total alkaloids in larkspur stalks treated with metribuzin to inhibit photosynthesis and control plants. The increase in concentration of toxic and total alkaloids in plants treated with metribuzin was relative to the decline in the biomass of the plant, presumably because soluble carbohydrates were depleted and not replenished through photosynthesis. The lack of difference in absolute amount of alkaloids between control and metribuzin-treated plants and the lack of change in absolute amount of alkaloids over the 14-day experiment suggests that photosynthesis inhibition did not increase alkaloid synthesis.

Our results differ from those reported by Wink and Witte (1984) dealing with quinolizidine alkaloids in annual lupine (*Lupinus albus*). Synthesis of quinolizidine alkaloids occurred in chloroplast of leaves and was driven by light. Alkaloid concentration in leaves increased as light intensity increased and peaked at midday. Metribuzin disrupts chloroplast membranes. If norditerpenoid alkaloid synthesis occurred in chloroplasts, we would have expected the alkaloid concentration to decline in plants treated with metribuzin. Since the alkaloid concentration increased (but the absolute amount of alkaloids remained constant), we conclude that norditerpenoid alkaloids are not synthesized in chloroplasts during the bud elongation growth stage, the time at which this experiment was conducted.

In larkspur plants stressed by three-day shade or dark treatments, the slight increase in toxic alkaloid concentration probably resulted from the relative decrease in nonstructural carbohydrates as they were utilized or translocated out of the leaves.

Long-term shade treatments on potted larkspur plants and larkspur plants growing under shade in the field had lower toxic alkaloid concentrations than larkspur plants growing in open sunlight. These plants were developmentally younger than those in the sun and should have had higher concentrations of alkaloids. It is feasible that reduced light and cooler temperatures in shaded environments may reduce synthesis of norditerpenoid alkaloids in larkspur early growth, resulting in lower levels of alkaloids as the plants mature.

The combined results of our experiments do not fit the carbon/nutrient balance theory of plant defense compounds (Bryant et al., 1983, 1992), which predicts that as photosynthesis declines, resources are shunted to synthesis of N-based defense compounds. Short- and long-term shade, dark stress, or photosynthesis inhibition apparently did not increase alkaloid synthesis. Furthermore, simulated storms (Ralphs, unpublished data) and insect damage from the larkspur mirid [*Hopplomachus affiguratus* (Heteroptera: Miridae)] (Ralphs et al., 1998) did not increase alkaloid levels. It appears that external environmental stresses do not affect norditerpenoid alkaloid synthesis in larkspur.

However, the concentrations of toxic alkaloids in tall larkspur decline dramatically over the growing season, form a high of 1.2% of dry weight in new early growth, to a low of 0.2% in mature plants (Ralphs et al., 1997). Gershenzon (1994) stated that the enzymes required for synthesis of some secondary compounds are only active for short periods in the plant's development. He reported monoterpene biosynthesis in peppermint (*Mentha piperita*) was restricted to a brief period during the first two weeks of leaf ontogeny. Enzymes for the synthesis of indole alkaloids in periwinkle (*Catharanthus roseus*) (DeLuca et al., 1988), and cyanogenic glycosides in sorghum (*Sorghum bicolor*) (Halkier and Moller 1989) were only present in early growth. Other studies reported early synthesis of secondary compounds, such as purine alkaloids from *Coffea arabica* (Frischknecht et al., 1986), alkaloid synthesis in *Cinchona* (Aerts et al., 1991),

caffeine in tea leaves (Fujimori et al., 1991), and glucoinsulates in rape leaves (Porter et al., 1991). Lerdau et al. (1994) cited several examples where monoterpene concentrations were highest in small expanding leaves. The results from these examples from the literature, as well as the results from our experiments, do not fit the growth/differentiation balance theory of plant defense (Lorio, 1986), in which secondary compound synthesis occurs during later periods of cell differentiation, not in the early growth stages of cell division and elongation.

We propose the hypothesis that norditerpenoid alkaloids in larkspur are synthesized during early growth and that synthesis slows down and stops as the plants continue to grow. The constant amount of alkaloids is then diluted as the biomass of the plant increases as it continues to grow, thus accounting for the observed decline in both toxic and total alkaloid concentration as the growing season progresses. The existing alkaloids in the plant may be translocated to the seeds as they mature, thus accounting for the observed increase in toxic alkaloids in pods (Pfister et al., 1994). Environmental conditions during this early growth period may influence the subsequent alkaloid level, but environmental changes throughout the rest of the season would not affect alkaloids. There still remains a substantial amount of variation in alkaloid content among plants. This may be due to genetics or to differences among microsites during the early growth period.

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PREMATING BEHAVIOR OF *Bombus confusus* MALES AND ANALYSIS OF THEIR LABIAL GLAND SECRETION

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Abstract—Premating behavior in the bumblebee *Bombus confusus* was studied. Visual searching for females is not the only premating strategy of this species, as was believed earlier. Males of *B. confusus* have a normally developed labial gland and its secretion is used to mark a perch from which they visually search for females. The labial gland secretion contains geranylcitronellol and (*Z*)-9-octadecenyl acetate as the main components.

Key Words—Hymenoptera, Apidae, *Bombus confusus*, bumblebees, perching behavior, marking pheromone, geranylcitronellol, (*Z*)-9-octadecenyl acetate.

INTRODUCTION

Bumblebee mating tactics have been studied by various authors and classified into several categories. However, these categories are not very distinct, with the most detailed modern classification published by Schremmer (1972) for Central European species. He recognized four categories of behavior displayed by bumblebee males, as follows: (1) sitting or flying around the nest entrance waiting for virgin females (Figure 1, group IA); (2) regular patrolling flights, usually performed in open areas but allowing the males to approach the nest entrance easily (Figure 1, group ICa); (3) regular patrolling flights away from the nest (Figure 1, group ICb); and (4) perching behavior—resting on a perch, darting at various passing objects, with no patrolling behavior exhibited (Figure

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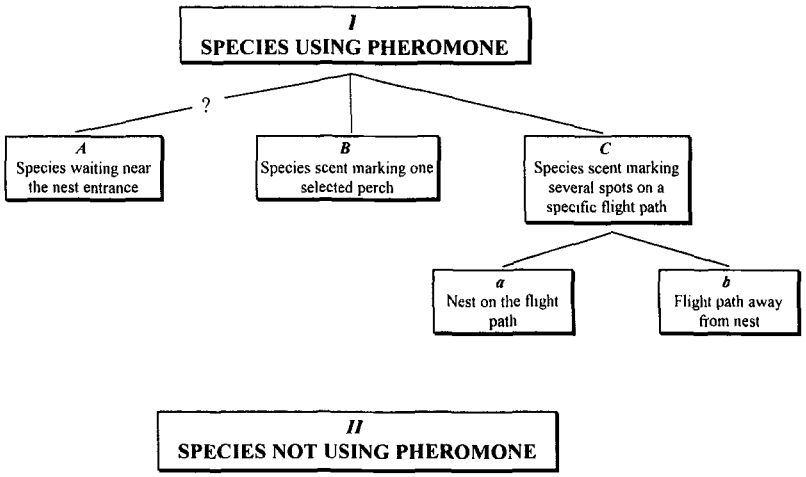


FIG. 1. Classification of the bumblebee premating behavior (with the use of pheromone selected as the basic criterion). The figure summarizes different literature sources (Schremmer, 1972; Svensson, 1980; Morse, 1982; Lloyd, 1981; Villalobos and Shelly, 1987).

1, groups IB and II). This classification has been used by many subsequent authors (Svensson, 1980; Morse, 1982; Free, 1987).

A similar system was proposed by Lloyd (1981), except he did not distinguish between Schremmer's behavioral types 2 and 3 above (groups ICa and b). More recently, Villalobos and Shelly (1987) recognized two main categories of male behavior and based their distinction on the location of female encounter sites. Males either wait by the nest entrance for virgin females or they search for queens away from the nest. Within the second category, the authors further observed that in searching for a female, males select perches or flight paths that they scent-mark to attract passing females, or males visually search for females from a prominent perch without the use of pheromones.

Recently, Williams (1991) described new types of mating tactics for Asiatic bumblebee species from Kashmir. His racing, territorial, and cruising behavior seem to be only a slightly modified perching behavior of group IB in Figure 1.

We have selected the use of pheromone as a basic criterion for classification. Group IA in Figure 1 is marked by a question mark because the use of pheromone has not been explicitly demonstrated for this group. Although the behavioral observations of *Bombus fervidus* (Fabricius, 1798) made by Lloyd (1981) indicated the use of pheromone, this was not confirmed by Villalobos and Shelly (1987) during their study of *Bombus sonorus* (Say, 1837). Recently, however, Bergström et al. (1996) analyzed the labial gland secretion of *B.*

sonorus and found compounds similar to pheromone components known to be present in other bumblebee species. This suggests the use of the secretion to attract females, which is why we tentatively include this group among species using pheromone.

B. confusus is usually presented as a typical representative of group II in Figure 1 in which perched males exclusively search visually for females (Schremmer, 1972; Morse, 1982). This species is reported to be a nonpatrolling, entirely visually oriented bumblebee, characterized by its enormous eyes. Although in other perching species of bumblebees that have large eyes scent-marking of their perching site has been observed (Haas, 1949; Alcock and Alcock, 1983), this is not known in *B. confusus* males, and Schremmer (1972) characterized them as obviously purely visually oriented. The labial gland of such a species would be expected to be reduced and nonfunctional. Contrary to our expectations, the dissected labial glands of all *B. confusus* males collected by us were normally developed, equally large or even slightly larger than those of the species exhibiting regular patrolling behavior. This result led us to analyze the chemical composition of the gland content. Neither the existence of a functional labial gland nor the chemical composition of its secretion has been described for this species in the literature before.

METHODS AND MATERIALS

Males (14 individuals) of *Bombus confusus* Schenck, 1859 were collected in August 1995 in the Central Mountains of the Czech Republic, at Oblík, elevation 509 m. Detailed observations of patrolling behavior took place in the same location during August and September 1996. Males were observed in four whole-day sessions. On the four days, respectively, 1, 3, 2, and 2 males were observed. Males were not marked to distinguish individuals.

For chemical analysis, living insects were collected and transported to the laboratory, then kept in the freezer until the labial glands were dissected. Each dissected gland was extracted with 50 μl of hexane, and each sample was analyzed separately.

Chromatography. The extracts were analyzed with a Hewlett-Packard HP 5890 gas chromatograph equipped with a split/splitless injector held at 220°C and a flame ionization detector at 290°C. Identifications of the components were made from gas chromatography–mass spectrometry (GC-MS) with an on-column injector and a mass detector (Fisons MD800) working in electron impact ionization mode. A DB-5 column (30 m × 0.25 mm) and carrier helium gas at 0.7 ml/min, measured at 50°C, were used for the separations. The temperature program started at 50°C after a 2-min delay, increased to 140°C at 70°C/min, then to 240°C at 2°C/min, and finally to 300°C at 5°C/min. For the GC-MS

analyses, a 15-m column of the same type (DB-5 ms) was used. The helium flow and the temperature program were slightly modified. The identification of compounds was based mostly on their mass spectra compared to those in the National Institute of Standards and Technology (NIST) Library and on the co-chromatography with synthetic or commercially available standards. The gas-phase infrared spectrum (GC-FTIR) of one of the isolated components was measured on an HP 5890 gas chromatograph coupled with an HP 5695A IRD equipped with a narrow-band (4000-750 cm^{-1}) infrared detector (mercury cadmium telluride).

Preparative Column Chromatography of Gland Extract. A selected hexane extract of one gland (50 μl) was chromatographed on silica gel (Merck 60, 0.040–0.063 mm; 290 mg) in a Pasteur pipet. The elution of a sample started with pentane, followed by hexane–ether mixtures (1–40% of ether). Two fractions contained pure compounds at high enough concentrations to enable further characterization and derivatization.

Derivatization and Preparation of Standards. The dimethyl disulfide (DMDS) adduct of isolated octadecenylacetate was prepared according to a published procedure (Attygalle et al., 1993). The same procedure was also done with the whole extract to allow the determination of the double-bond positions in the minor components. A standard of geranylgeraniol (Sigma) was acetylated by acetic anhydride in pyridine by a standard procedure. Geranylcitronellol was synthesized in our laboratory earlier (Valterová et al., 1996). A sample of geranylcitronellal was obtained from the oxidation of geranylcitronellol by pyridinium chlorochromate in dichloromethane. Standards of geranylcitronellyl hexanoate and geranylcitronellyl octanoate were prepared from geranylcitronellol and the respective acids via the corresponding chlorides. 1,1-Dichloromethylmethyl ether (50 μl) was added to a vial containing hexanoic or octanoic acid (10 μl), and the mixture was stirred for 2 hr. Afterward, the excess 1,1-dichloromethylmethyl ether was evaporated. Geranylcitronellol (1 μl) was dissolved in 100 μl pyridine, and the solution was cooled to 0°C and added to the vial with the residue of the chloride from the previous reaction. The reaction mixture was stirred for 1 hr at room temperature, water was added, and the resulting ester was extracted with ether. The ether layer was dried and analyzed by means of GC-MS.

RESULTS

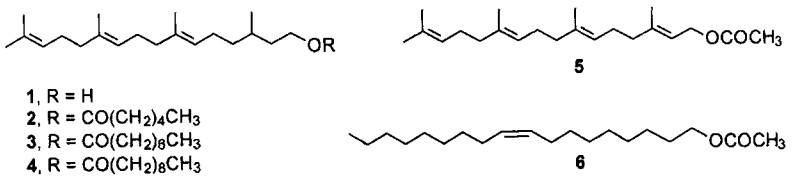
Biological Observations. The males' perches were situated in an elevated area, near the top of the hill at Oblík. Males (two to three individuals) were sitting on perches in the same area, separated by a distance of 40–60 m. No interactions between males were observed. The males were not marked; there-

fore, we have no information about the possible repeated use of the same perch on different days. During our observations, no queens were seen in the area.

Males of *B. confusus* have a fully developed and functional labial gland (this study), and the gland produces a secretion used for marking a site where a male is waiting for a female. Thus, we offer here our interpretation of premating behavior of *B. confusus*: The male selects a perch on any elevated site and marks it with his pheromone. He marks only one point or several points in very close proximity. The male spends all day on this elevated perch waiting for a passing female that would be attracted by the pheromone. To increase the probability of a successful meeting with a female, the male has secondarily developed a very conspicuous type of behavior that involves flight toward any object resembles a female by its size flying within 10–15 m of the perch. A characteristic trait of the males is their enormous eyes. The enlargement of the eyes, a very striking morphological adaptation, seems likely to aid the males in recognizing flying objects, and potential mates, at a long distance.

Indirect proof of our interpretation of the use of a pheromone by *B. confusus* males is the fact that males made most of their pursuit flights downwind (73%, $N = 271$; quantitative data based on nine 10-min observation periods of two males). They reacted most often to insects flying upwind, a behavior suggesting that the flying females were following a scent trail in the air. However, this observation needs further confirmation because the direction taken in our limited observations could have been adversely influenced by the configuration of the terrain. Schremmer (1972) did not find any preference in the direction of the males' pursuit flights.

Chemical Analysis of Labial Gland Secretion. The labial gland secretion from 12 male samples contained 23 compounds; mean ± SD data are presented in Table 1 and structures of the principal components are shown in Figure 2. Samples from different individuals were similar in chemical composition. The two most abundant components, octadecenyl acetate and geranylcitronellol, were isolated by micropreparative column chromatography. Octadecenyl acetate eluted with a mixture of hexane–ether (95:5), while geranylcitronellol formed the



1, R = H
2, R = $CO(CH_2)_4CH_3$
3, R = $CO(CH_2)_8CH_3$
4, R = $CO(CH_2)_8CH_3$

FIG. 2. Main components identified in the labial gland secretion of *Bombus confusus* males.

major part of a fraction eluted with hexane–ether (7:3). Retention time and mass spectrum of geranylcitronellol isolated from *B. confusus* were identical with those of the standard sample prepared in our laboratory earlier (Valterová et al., 1996).

Identification of octadecenyl acetate was based on its mass spectrum. The position of the double bond was determined by GC-MS analysis of the dimethyl disulfide adduct. The fragments 173 and 231 (*m/z*) showed that the double bond is located in position 9. The structure was confirmed by coinjection with a standard of 9-octadecenyl acetate. Although we had both (*E*)- and (*Z*)-9-octadecenyl acetates available, we did not succeed in chromatographic separation of the two isomers in order to determine the geometry of the double bond. The *Z* configuration of the double bond is based on the measurement of infrared spectrum in the gas phase (Attygalle et al., 1994). The presence of the 3012 cm^{-1} band (= C–H stretch), and the absence of a band at 890 cm^{-1} (*E*-wag) established the configuration of the double bond in our isolated sample as (*Z*)-9-octadecenyl acetate.

Other compounds of the isoprenoid type were found as medium or minor components. Geranylgeranylacetate and geranylcitronellal were identified by cochromatography with synthetically prepared standards. Six esters of geranylcitronellol were detected in the secretion. Geranylcitronellyl acetate formed a minor component. Its structure was confirmed by comparison with a standard isolated earlier from *Psithyrus vestalis* (Valterová et al., 1996). Geranylcitronellyl hexanoate (1.6%) and geranylcitronellyl octanoate (2.9%) were identified on the basis of their mass spectra, and their structures were confirmed by comparison of retention times and mass spectra with those of synthetically prepared standards. Free hexanoic and octanoic acids, found in the secretion in small proportions, might be precursors of the esteric pheromone components. Geranylcitronellyl decanoate, together with traces of geranylcitronellyl dodecanoate and geranylcitronellyl tetradecanoate, was also detected in the gland extracts.

Methyl octadecenoate, ethyl octadecenoate, and octadecenol formed trace components of the secretion. They could not be separated preparatively from other secretion components and isolated in a pure state. However, we were able to determine the positions of the double bonds in these compounds from the mass spectra of DMDS adducts prepared in a mixture with other components. The fragment at *m/z* 173 and the corresponding fragments at *m/z* 217 (M^+ 390), 231 (M^+ 404), and 189 (M^+ 362) in methyl octadecenoate, ethyl octadecenoate, and octadecenol, respectively, proved the location of the double bond in position 9 in all three compounds. The configurations of the double bond could not be determined because there was too little of these compounds in the mixture.

The unsaturated hydrocarbons (C_{25} and C_{27}) had the double bond located in position 7. In the case of pentacosene, two more isomers were detected, 9-pentacosene (0.16%) and 8-pentacosene (threshold detection quantity).

TABLE 1. COMPOUNDS FOUND IN LABIAL GLAND OF *Bombus confusus*, THEIR MASS SPECTRA, AND METHOD OF IDENTIFICATION

Compound[a]	Retention time (min)[b]	Relative proportions (%, mean ± SD)[c]	Mass spectral fragments (*m/z*)	Method of identification
Hexanoic acid	5.99	2.47 ± 2.12	43, 60, 73, 87, 101	GC-MS
Octanoic acid	7.56	0.88 ± 1.36	43, 60, 73, 101, 115, 129	GC-MS
9-Octadecen-1-ol	37.13	0.24 ± 0.14	31, 41, 55, 67, 82, 96, 222, 250	GC-MS, DMDS-adduct
Geranylcitronellal	38.36	0.47 ± 0.21	41, 69, 81, 95, 221, 247, 290	GC-MS, coinjection with standard
Methyl 9-octadecenoate	39.04	1.74 ± 1.38	59, 69, 74, 87, 180, 222, 264	GC-MS, DMDS-adduct
Geranylcitronellol, **1**	41.82	29.94 ± 4.89	41, 69, 81, 95, 223, 249, 292	GC-MS, coinjection with standard
Ethyl 9-octadecenoate	42.09	0.68 ± 1.75	41, 55, 88, 101, 222, 264, 310	GC-MS, DMDS-adduct
(*Z*)-9-Octadecenyl acetate, **6**	43.04	47.71 ± 8.45	43, 55, 61, 82, 250, 267, 310	GC-MS, DMDS-adduct, GC-IR, coinjection with standard
Ethyl octadecanoate	43.26	0.15 ± 0.27	43, 88, 101, 157, 213, 269, 312	GC-MS
Octadecyl acetate	43.89	0.03 ± 0.06	43, 61, 69, 83, 224, 252	GC-MS
Geranylcitronellyl acetate	45.12	0.02 ± 0.08	41, 69, 81, 95, 265, 291, 334	GC-MS, coinjection with standard
Geranylgeranyl acetate, **5**	46.32	1.69 ± 0.40	41, 69, 81, 93, 263, 289, 332	GC-MS, coinjection with standard
Tricosane	47.81	3.14 ± 1.00	57, 71, 85, 99, 113, 324	GC-MS
Pentacosadiene	54.38	0.81 ± 0.21	41, 55, 69, 82, 96, 348	GC-MS
9-Pentacosene	54.96	0.16 ± 0.17	43, 55, 69, 83, 97, 350	GC-MS, DMDS-adduct
7-Pentacosene	55.23	1.07 ± 0.31	43, 55, 69, 83, 97, 350	GC-MS, DMDS-adduct
Pentacosane	55.87	1.26 ± 0.52	57, 71, 85, 99, 113, 352	GC-MS
Geranylcitronellyl hexanoate, **2**	59.82	1.61 ± 0.61	69, 81, 95, 99, 321, 347, 390	GC-MS, coinjection with standard
Heptacosadiene	60.41	0.16 ± 0.13	43, 55, 69, 82, 96, 376	GC-MS
7-Heptacosene	61.10	0.10 ± 0.10	43, 55, 69, 83, 97, 378	GC-MS, DMDS-adduct
Heptacosane	61.54	0.01 ± 0.03	57, 71, 85, 99, 113, 380	GC-MS
Geranylcitronellyl octanoate, **3**	64.26	2.89 ± 1.62	69, 81, 95, 127, 349, 375, 418	GC-MS, coinjection with standard
Geranylcitronellyl decanoate, **4**	76.57	2.79 ± 3.88	69, 81, 155, 362, 377, 403, 446	GC-MS

[a]The compounds are listed in elution order on a 30-m DB-5 column (5% phenyl methyl silicone).
[b]Temperature program: 50°C (2 min); 70°/min to 140°C; 2°/min to 240°C; 5°/min to 300°C; helium flow 0.7 ml/min at 50°C.
[c]Mean values calculated from 12 samples; SD = standard deviation.

Although small amounts of the C_{25} and C_{27} dienes were present (Table 1), their DMDS adducts could not be found and double bond positions could not be determined.

DISCUSSION

An important finding is that all the C_{18} unsaturated compounds found in *B. confusus* have the same position 9 for their double bond. The position of the double bond and its configuration in octadecenyl acetate were in agreement with biosynthetic data published in the literature (Lanne et al., 1987). All unsaturated compounds isolated from bumblebees so far have the *Z* configuration of double bonds, and it is always located in odd-numbered positions of the aliphatic chain.

Geranylcitronellol is one of the most common components of labial gland secretions of bumblebees. It has been previously found in other species of bumblebees and cuckoo bumblebees, such as *Bombus terrestris* (Kullenberg et al., 1970; Bergström et al., 1981), *Bombus hypnorum* (Kullenberg et al., 1970; Svensson and Bergström 1977), *Bombus lapponicus* (Bergström and Svensson 1973), *Bombus hyperboreus* and *Bombus balteatus* (Svensson and Bergström 1979), *Psithyrus rupestris* (Kullenberg et al., 1970; Cederberg et al., 1984), and *Psithyrus vestalis* (Valterová et al., 1996). It has also been detected in some other bumblebee species as a minor component of their labial gland secretions. In addition, geranylcitronellol forms the main component of the labial gland secretion in the American cuckoo bumblebee *Psithyrus insularis* (Bergström et al., 1996). Geranylcitronellol was also identified in *B. hypnorum* workers (Ayasse et al., 1995).

(*Z*)-9-Octadecenyl acetate was previously found as a labial gland component of *Bombus muscorum* (Appelgren et al., 1991) and *Bombus sylvarum* (Lanne et al., 1987). It also is produced by workers of *B. hypnorum* (Hefetz et al., 1993) and *B. vorticosus* (Tengö et al., 1991) in the Dufour gland.

Geranylcitronellyl hexanoate and geranylcitronellyl octanoate have not been previously detected in labial gland secretions of any bumblebee species. A question arises about the function of these "heavy" compounds in the secretion, as they are not volatile and can hardly serve as a signal for females from a distance. So far, some of the long-chain geranylcitronellyl esters were reported as components of Dufour gland secretion, but never from the labial glands of males. For example, farnesyl hexanoate was found in *B. terrestris* workers (Tengö et al., 1991). A group of homologous esters of geranylcitronellol with acids of the chain length C_{10}–C_{20} was isolated from different glands of *B. hypnorum* workers (Ayasse et al., 1995).

In addition to the identification of the labial gland components, we tested whether the secretion is used for marking the perch. The perches used by the bumblebee males were found to be old dry yarrows (*Achillea* sp.) or dead dry corn flowers (*Cyanus* sp.). These plants, growing in small clusters, were rather

abundant in the vicinity. The ones that served as the perches and were marked by the male secretion had a distinct smell detectable even by humans, while the ones that were not used by the bumblebees were odorless. The smell was identical to that of the dissected males. Identification of the chemical components of the mark deposited on the dry plant perches is planned for future studies.

Field observation of premating behavior of *B. confusus*, together with our discovery of a functional labial gland whose secretion is used for marking, enables us to make an important inference. Based on these results, we have concluded that *B. confusus* has been considered incorrectly as a species not using pheromone, and using only visual recognition during premating behavior (Figure 1, group II). We suggest that group II bumblebees (species that do not use pheromones) may not exist at all. What we have shown is that *B. confusus* does not, in fact, differ from other perching bumblebees that use a selected, scent-marked site (Figure 1, group IB). The females probably are attracted by the emitted labial gland secretion, and the final recognition of the female is visual. The male is adapted with exceptionally large eyes that probably aid in vision.

The premating behavior of *B. confusus* as described by us is the same as that of the Nearctic species *Bombus nevadensis auricomus* (Robt., 1903) and *Bombus griseicolis* (De Geer, 1773) studied by Alcock and Alcock (1983). The males of both these species are also characterized by bulging or swollen eyes (Milliron, 1971, 1973), and they were observed to scent-mark spots near their perches. Villalobos and Shelly (1987) placed them into the same group as species scent-marking regular flight paths (patrolling species).

B. nevadensis seems to be phylogenetically very closely related to *B. confusus*. Both species are considered to be among the earliest diverging bumblebees species surviving into the present time (Williams, 1994). The premating behavior and enlarged eyes are common to several bumblebee species belonging to different subgenera. The characteristic premating behavior and large eyes may have evolved independently several times during the speciation of bumblebees.

The presence of a labial gland secretion and the use of pheromones seem to be common characteristics of all recent *Bombus* (Latreille, 1802) and *Psithyrus* species (Lepeletier, 1833) species. Most probably, it is a primary, ancestral way of attracting females within these genera. Differences between the species exist in the location of female encounter sites, in the number of scent-marked spots, and in the degree of visual orientation. Visual orientation is probably always present to some extent during interactions between males and females of all bumblebees species (Free, 1971, 1987; our own observation), with the exception of those cases when copulation takes place inside the nest.

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CHEMICAL ANALYSIS OF SOILS OF KOWLOON (HONG KONG) EATEN BY HYBRID MACAQUES

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Abstract—Geophagy, the deliberate act of eating soil, has been observed in various primate species, although the reasons for this behavior are poorly understood. The present study supports the general hypothesis that the physical and/or chemical nature of the soil is a likely reason for geophagy in hybrid macaque monkeys of Kowloon, Hong Kong. Samples were taken from areas where macaques were seen to be eating soil and from areas where there was no evidence of geophagy. Samples that were refused by the macaques were also obtained. Soils were analyzed for the following chemical and physical properties: soil pH, cation exchange capacity, organically bound and inorganic crystalline and poorly crystalline Fe and Al, organic carbon, acid extractable elements, particle size, and soil mineralogy. The study supports a hypothesis that the monkeys are using visual cues to choose preferentially soils for ingestion. More frequently they chose fine-textured soils that were higher in Fe and Al oxides. We speculate that these properties are sought to aid in digestive processes.

Key Words—Geophagy, soil ingestion, macaque, soil geochemistry and mineralogy, Hong Kong, primate.

INTRODUCTION

Geophagy, the deliberate act of eating soil, has been observed in various nonhuman primate species (Fossey, 1983; Ganzhorn, 1987; Davies and Baillie, 1988; Mahaney et al., 1993). While geophagy has been investigated in various species, there is, as yet, no general consensus as to the reasons for this behavior. Many hypotheses have been formulated that involve either potential physio-

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chemical functions provided by the soil in the animal gut or the actual physical qualities of the soil.

The hypothesis that the soil is eaten in order to fulfill nutritional requirements has been studied extensively. Researchers have speculated that soil is eaten because of the iron, sodium, magnesium, and/or bromine content (Mahaney et al., 1993; Lindburg, 1977; Oates, 1978). However, Johns and Duquette (1991) contend that there is little evidence to suggest that either iron or sodium are sought by most animals. Other researchers question the importance of mineral supplementation (Hladik and Gueguen, 1974; Oates, 1978). Robbins (1993) cautions that, while nutritionally significant amounts of minerals are present, soil may be consumed for other reasons. He speculates that soil clay may be involved in metal retention, thereby preventing metal absorption by the animal.

The clay fraction may also adsorb some ingested organic plant toxins (tannins, alkaloids, oxalates), resulting in their removal (Oates, 1978; Hladik, 1977; Wrangham, 1987; Gilman et al., 1985; Johns, 1986). Kaolinite is one of the minerals capable of this adsorption (Said et al., 1980; Jones, 1957; Daykin, 1960), and the presence of kaolin in the clay fraction of soils has been implicated in geophagy (Mahaney et al., 1993, 1995, 1996). It has long been known that kaolin aids in the treatment of gastric upsets (Gilman et al., 1985; Vermeer and Ferrell, 1985). Clays may also act in maintaining the pH of the gut.

Other hypotheses emphasize the physical, and not the physicochemical, qualities of the soil. Hladik and Gueguen (1974) suggest that the primates eat soil for the "tactile sensation in the mouth" (Hladik and Gueguen, 1974). However, Heymann and Hartmann (1991) do not believe this applies to tamarins because of the variable physical appearance of their soil samples, and thus, the tactile stimulus.

A final hypothesis considers geophagy as strictly behavioral; however, it has not been demonstrated as the sole reason (Mahaney et al., 1993). Behavioral tradition has been attributed to caprophagy (Harcourt and Stuart, 1977) and to ant-eating among gorillas (Watts, 1989) and does receive support from Cambefort (1981) and Watts (1989).

The present study adds to the body of evidence that supports the general hypothesis that the physical and/or chemical nature of the soil is likely the reason for geophagy. This study specifically involves a group of hybrid macaque monkeys living in Kowloon, Hong Kong. Samples were taken from areas where macaques were seen to be eating soil and from areas where there was no evidence of geophagy. The data collected are important in that samples that were physically refused by the macaques were also obtained. Very few of the studies to date have allowed for the comparison of soil properties from soils that are considered acceptable and unacceptable to the animal. We believe this makes this particular study unique.

METHODS AND MATERIALS

Field Area and Study Animal. Hong Kong, with a total area of 1045 km^2, lies south of mainland China and consists of the Kowloon Peninsula and many surrounding islands, including Hong Kong island. The combined effects of warm climate and monsoons in Hong Kong have led to highly weathered soils. Lumb and Fan (1975) found that the clay-size fraction of the Hong Kong soils they studied contained predominantly quartz and that in the extensively weathered soils kaolinite comprised up to 35% of the clay fraction. The highly weathered soils have a red or yellow color because of the oxidation of trace amounts of iron (Lumb and Fan, 1975).

The study area, Kam Shan Park, is contained within a 700-ha conservation area located in the Kowloon Peninsula (Burton and Chan, 1987, 1989). More than 200 macaques live in the park and are hybrids of various species. The most prevalent morphotype is that of the rhesus monkey (*Macaca mulatta*). The long-tailed macaque (*Macaca fasicularis*) is also common. The other species in the hybrid group are the Japanese macaque (*Macaca fuscata*), the bear macaque (*Macaca thibetana*), and the pigtailed macaque (*Macaca nemestrina*) (Burton and Chan, 1996).

Soil Sites and Sampling Procedure. During the two-week study period in February 1995, the macaques were observed eating soil at sites throughout the study area (Figure 1). Each site was less than a meter from the top of an eroded slope and plant roots were abundant. The soils at each of these sites had visual signs of scratching and, at some locations, the macaques had actually excavated caves, the largest being 100 cm wide, 45 cm high, and 60 cm deep. Nineteen samples were obtained from these sites and these samples are designated as "eaten" (E1–E19).

Four soil samples were taken from sites that did not show evidence of soil eating. Three of these sites were approximately 30 cm above a site where monkeys were observed eating soil, and one was 30 cm below. These sites did not have scratched surfaces and showed no sign of excavation. The soils tended to have a lighter color than those at nearby eaten sites. These soils are designated as the control samples (C1–C4).

Three soil samples were also obtained after a monkey was observed inspecting the soil (i.e., picking it up and smelling it) and subsequently discarding it. These samples were of lighter, duller color than the eaten and control samples and are designated as "refused" (R1–R3).

Samples of approximately 100 g were obtained using stainless steel implements and subsequently stored in plastic bags at 8–10°C.

Soil Analyses. All of the samples were air dried prior to analysis. The pH of the soils was measured in a 1 : 1 soil-to-water ratio (Hendershot et al., 1993).

The cation exchange capacity (CEC) of the soils was determined, following the method of Evans (1982), on all soils with the exception of five samples for which insufficient sample was obtained. Values for extractable iron and aluminum were obtained with three techniques: dithionite citrate (Carter, 1993) for total oxides, hydroxides, and oxyhydroxides of iron; acid ammonium oxalate (McKeague, 1978) for poorly crystalline Fe and Al oxide minerals; and sodium pyrophosphate (McKeague, 1978) for organically bound Fe and Al. Organic carbon was determined with a Leco Induction Furnace.

Extractable Fe and Al contents were converted to three fractions in the following manner (Evans and Wilson, 1985): the organically bound Fe and Al fractions, Fe_{or} or Al_{or}, were considered to be equivalent to the pyrophosphate-extractable fraction; the inorganic poorly crystalline Fe and Al fractions, Fe_{pc} and Al_{pc}, were calculated from the difference between oxalate and pyrophosphate-extractable fractions; and the inorganic crystalline Fe fraction, Fe_c, was calculated from the difference between dithionite and oxalate-extractable fractions.

Acid extracts were obtained by adding 10 g of water to 2 g of soil and titrating the suspension to pH 2 with 0.1 M HCl (Sheppard et al., 1995). A pH of 2 was chosen because it is the approximate pH of a monkey's stomach (Johns and Duquette, 1991). Acid suspensions were filtered, and the solution was subsequently analyzed for 18 major and minor elements by inductively coupled plasma–mass spectrometry (ICP-MS).

Particle size analysis was not completed due to small sample size. Some larger samples were wet sieved through a <250-μm sieve in order to determine the percentage of the coarse sand fraction and the combined percentage of the fine sand, silt, and clay fractions (International Soil Science Society Classification) (Gee and Bauder, 1986).

Soil and clay mineralogy was determined by X-ray diffraction (XRD) for six samples (two each of eaten, control, and refused). Random powder presses were used for total soil analysis and oriented glass slides were used in order to differentiate phyllosilicates in the clay size fraction (Whittig and Allardice, 1986). The clay size fraction was separated via sedimentation, and the clays were treated with dithionite citrate prior to XRD in order to remove Fe oxide coatings.

RESULTS AND DISCUSSION

The refused soil samples were observably different from the eaten and control samples in color and texture. While eaten and control samples were red or orange in color, the refused samples were quite grey and were much coarser textured than were the other samples. The organic carbon contents were very small, $<0.3\%$ and did not differ significantly in the eaten, control, and refused samples.

Soil pH ranged from strongly acidic (4.28) to slightly acidic (6.59) (Table 1). Although the mean pH for the control soils was the highest and that of the refused the lowest, there was great variation within each category, and mean pH values were not significantly different ($P > 0.05$).

While it was possible to analyze only 14 samples for percentage of coarse sand, the refused samples appeared to be much coarser textured than either the eaten or control samples. Eaten and control samples had coarse sand fractions that ranged from 22.0 to 33.1%, and the two analyzed refused samples had coarse sand fractions of 45.8 and 62.6% (Table 1 and Figure 1). Due to small sample size, we were unable to test for significances.

The eaten samples contained significantly greater amounts of inorganic,

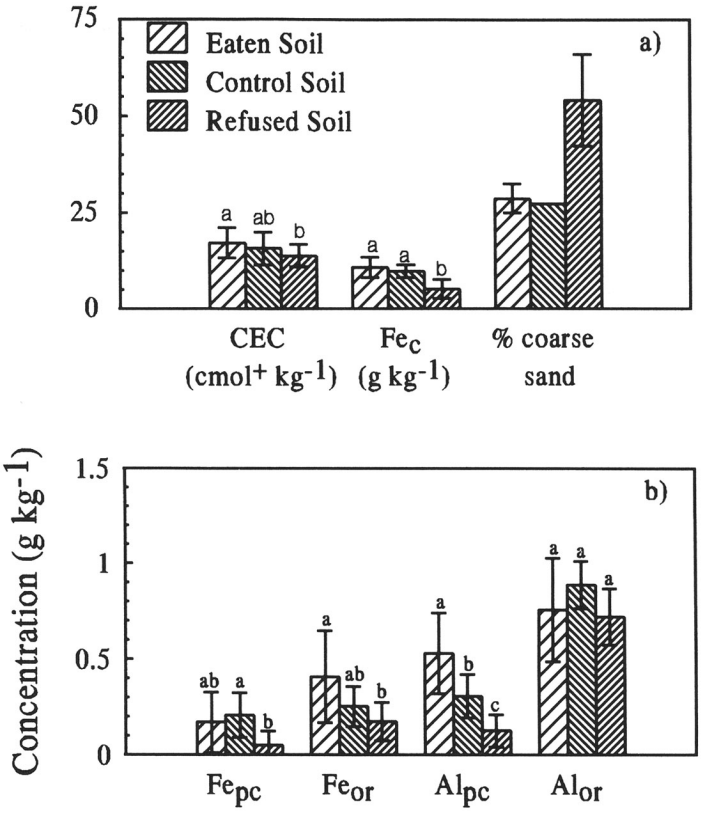

FIG. 1. Variation in soil properties between the eaten, control, and refused samples. Values within one property denoted by the same letter are not significantly different (t test, 0.05 level).

TABLE 1. CHEMICAL AND PHYSICAL PROPERTIES OF KOWLOON SOIL SAMPLES[a]

Sample	pH	CEC ($cmol_c/kg$)	Amount (g/kg^{-1})					% organic carbon	% coarse sand
			Fe_c	Fe_{pc}	Fe_{or}	Al_{pc}	Al_{or}		
E1	5.39	21.25				0.360	1.308	0.209	24.602
E2	5.35	19.88	11.28	0.000	0.300			0.216	IS
E3	6.59	13.63	6.94	0.241	0.315	0.831	0.584	0.229	32.574
E4	4.28	15.54	6.66	0.132	0.151	0.453	0.786	0.241	22.009
E5	4.88	IS	8.81	0.097	0.139	0.341	0.712	0.192	IS
E6	4.52	IS	8.79	0.023	0.175	0.251	0.883	0.211	IS
E7	6.16	15.21	7.09	0.150	0.149	0.673	0.323	0.201	32.87
E8	5.38	IS	7.24	0.000	0.881	0.510	0.678	0.241	IS
E9	4.46	16.19	14.93	0.228	0.131	0.676	0.578	0.218	IS
E10	4.04	18.03	12.34	0.141	0.612	0.572	0.674	0.210	31.371
E11	4.56	18.39	12.19	0.346	0.468	0.747	0.642	0.234	IS
E12	4.65	13.35	12.34	0.162	0.602	0.624	0.627	0.232	27.51
E13	4.77	IS	11.42	0.218	0.630	0.657	0.828	0.197	IS
E14	4.54	IS	12.69	0.028	0.682	0.560	0.896	0.212	IS
E15	4.58	17.24	11.54	0.247	0.347	0.389	0.960	0.213	28.20
E16	4.93	16.85	11.08	0.483	0.516	0.229	1.291	0.226	33.09
E17	4.41	19.28	13.92	0.426	0.366	0.519	0.859	0.200	25.80
E18	4.88	18.26	11.51	0.060	0.702	0.590	0.878	0.227	31.376
E19	5.09	16.19	14.22	0.000	0.187	0.335	0.474	0.216	26.659
C1	5.49	17.86						0.225	IS
C2	6.48	16.21	7.86	0.146	0.226	0.183	1.012	0.206	IS
C3	4.84	10.93	10.85	0.122	0.154	0.374	0.772	0.203	IS
C4	4.43	19.07	11.02	0.356	0.380	0.368	0.881	0.205	27.39
R1	5.99	11.62	6.94	0.000	0.251	0.158	0.607	0.200	45.842
R2	6.20	12.70	6.52	0.000	0.223	0.194	0.869	0.223	IS
R3	4.96	16.68	1.97	0.148	0.047	0.041	0.632	0.200	62.588

[a] E = eaten; C = control; R = refused; IS = insufficient sample.

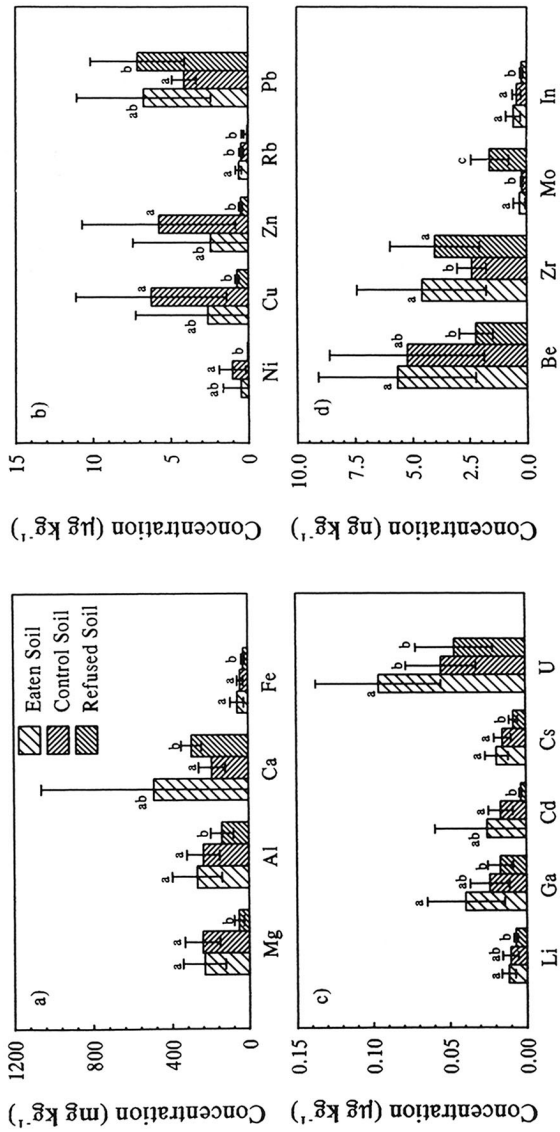

FIG. 2. Variation in elemental concentration between the eaten, control, and refused samples. Values within one element denoted by the same letter are not significantly different (t test, 0.05 level).

poorly crystalline aluminum than did the control ($P < 0.017$) or the refused ($P < 0.001$) samples (Figure 1). Values ranged from 0.041 g/kg (refused) to 0.831 (eaten) g/kg (Table 1). There were no significant differences between samples for organic complexed aluminum, which ranged from 0.323 to 1.308 g/kg (Figure 1).

Inorganic crystalline iron comprised on average 95% of the total iron, and samples contained from 1.97 g/kg soil (refused) to 14.93 (eaten) g/kg soil (Table 1). The amount of crystalline iron in the refused samples was significantly lower than in the eaten ($P << 0.001$) and control ($P < 0.002$) samples (Figure 1). All of the values obtained for inorganic, poorly crystalline and organic complexed iron were less than 1 g/kg, and there were only significant differences between control and refused samples (Figure 1). The eaten and refused samples contained significantly different quantities of organic complexed iron ($P < 0.03$) (Figure 1).

The cation exchange capacity (CEC) of the soils varied from 10.93 to 21.25 $cmol_c/kg$ (Table 1). The CEC of the eaten soils was significantly greater ($P < 0.030$) (Figure 1) than that of the refused soils, indicating that the buffering capacity and the adsorptive capacity of the eaten soils was also higher.

The buffering capacity of the soil is important with regards to buffering the stomach against fatty acids produced during ingestion (Davies and Baillie, 1988). The magnitude of CEC is related to soil texture, clay mineralogy, organic matter content, and the quantity of Fe and Al oxides. While all soil samples had very low organic carbon contents, the percentage of coarse sand was lower and oxides of Fe and Al (inorganic crystalline Fe and inorganic, poorly crystalline Al) were significantly higher in the eaten samples than in the refused samples, which agrees with the CEC findings.

There was a great deal of variability in the acid extraction data (standard deviations were very large), and much larger sample numbers are needed. However, some statistically significant differences were found (Figure 2). The eaten samples contained significantly greater amounts of Mg ($P < 0.001$), Cs, Rb, and In ($P < 0.01$), and Fe, Li, Be, and Al ($P < 0.05$) than the refused samples. A shortage of magnesium in the monkey's diet is not expected since chlorophyll contains magnesium and is abundant in plants (Robbins, 1993). Iron is an important element in biological systems because it is a metal chelate of hemoglobin and myoglobin and a component of many enzymes (Robbins, 1993). The acid-extractable Fe represented only a very small portion of the Fe associated with minerals and organic matter (Figure 1), and most of the "solid" Fe was not dissolved at these low pH values.

The eaten samples were found to be significantly higher than the control samples in Rb, Zr, and Mo ($P < 0.05$). The refused samples contained significantly higher amounts of Mo than did the eaten samples ($P < 0.001$). While

molybdenum is an essential element, it is uncommon for animals to be deficient in it. In larger amounts it can cause copper deficiencies and the "ability of wildlife to purposefully select low-molybdenum feeds would reduce the chances of toxicity" (Robbins, 1993, p. 67). The refused samples also contained significantly higher amounts of Mo ($P < 0.001$), Ca ($P < 0.01$), Pb and Zr ($P < 0.05$) than did the control samples. The control samples contained significantly higher amounts of Mg ($P < 0.001$), Cd ($P < 0.01$), and Cs, Ni, Cu, Zn, In, Al, and Fe ($P < 0.05$) than the refused samples.

Other researchers have suggested that geophagy provides monkeys with increased Na intake (Mahaney et al., 1993). However, in this study, no significant differences in Na content were found between eaten, control, and refused samples.

XRD traces for each of the soil samples indicated a dominance of kaolinite and quartz with trace amounts of gibbsite and ferrihydrite. Trace peaks associated with a feldspar, possibly albite, were found in the control and, more dominantly, in the refused samples. There was no evidence of feldspars in the eaten samples. The clay fractions of all the soils sampled were dominated by kaolinite with trace amounts of gibbsite. A very small 1.4-nm peak was also identified, which indicated the presence of a 2:1 phyllosilicate mineral.

Since all of the samples were dominated by kaolinite, this does not provide a basis for the preferential eating habits of the monkeys. However, trace amounts of feldspar in the refused and control samples indicate soils that may be less weathered than the eaten soils. While we are not suggesting that the monkeys have clay mineralogical abilities, they may be selecting more weathered soils based, again, on their redder color and increased Fe oxide content. It is interesting to note that the presence of kaolinite and Fe oxides would provide chemically different adsorbing surfaces in the very acidic monkey gut. At a pH value of approximately 2, kaolinite surfaces would be negatively charged and could provide sites for the sorption of both metals and organic bases such as various alkaloids. However, Fe oxides would be positively charged in this acidic environment and could therefore provide sites for sorption of anionic plant toxins such as oxalates.

CONCLUSIONS

This study provides results that enable us to speculate on some possible reasons for geophagy in macaque monkeys. It is reasonable to assume that the monkeys are physiologically capable of choosing some soils and refusing others strictly on the basis of either sight, smell, or feel. Hence soil texture and crystalline Fe content offer tactile and visual cues which may enable the monkeys

to preferentially choose soils for ingestion. They more frequently chose finer textured soils that were higher in Fe and Al oxides. While we do not yet know the exact reasons for this preference, we speculate that these properties are sought to aid in digestive processes including buffering the pH of the stomach and providing adsorbing surfaces for various toxins. These soils may also be a source of nutritionally essential Fe.

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SCENT-MARKING IN THE EURASIAN BEAVER (*Castor fiber*) AS A MEANS OF TERRITORY DEFENSE

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Abstract—Beaver (*Castor* spp.) normally scent-mark by depositing castoreum and/or anal gland secretion on scent mounds close to the water's edge. The aim of this study was to investigate the hypothesis that the Eurasian beaver (*C. fiber*) scent-marks as a means of territory defense. Scent-marking behavior was studied during an entire year (April 1, 1995–March 31, 1996) in seven adjacent territories along 9.2 km of the Bø River in Telemark County, Norway. The number and location of fresh scent marks were recorded biweekly. The main results showed that: (1) the number of scent marks in territories was significantly higher in spring, when dispersal of subadults normally occurs than during the rest of the year; (2) the number of scent marks was clumped near territorial borders; and (3) the number of scent marks was significantly greater upstream than downstream of the lodge.

Key Words—Beaver, *Castor fiber*, *Castor canadensis*, castoreum, anal gland secretion, scent mound, scent-marking, territorial behavior, Norway.

INTRODUCTION

Communication and social recognition in many mammals are based on olfactory signals (Wynne-Edwards, 1962; Ralls, 1971; Schulte et al., 1994). Scent marking has high persistence and is effective even in the absence of the sender (Wynne-Edwards, 1962; Bollinger, 1980). Olfactory communication can be defined as the process whereby a chemical signal is generated by a presumptive sender and transmitted (generally through the air) to a presumptive receiver, who, by means of adequate receptors, can identify, integrate, and respond (either behaviorally or physiologically) to the signal (Eisenberg and Kleimann, 1972).

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Mammalian scent-marking is associated with territorial defense (Gosling, 1990). It is widely accepted that mammals scent-mark their territories to advertise their occupancy and ownership of the territory (Peters and Mech, 1975; Macdonald, 1980; Erlinge et al., 1982; Gosling, 1982; Gorman and Mills, 1984; Smith et al., 1989), but it is still under debate how scent marks actually function in terms of territory maintenance (Gorman, 1990; Gosling, 1990; Richardson, 1991). For many years it was believed that scent marks help deter intruders from entering a territory or at least to intimidate them (Hediger, 1949; Geist, 1964; Johnson, 1973). Although scent marks are unlikely to exclude totally all intruders from exploiting resources within a territory, they may limit the degree (in time and space) to which the territory is intruded, and hence indirectly protect its resources. The fact that not all territorial intruders are intimidated has stimulated the search for new explanations as to how scent marks function in territory maintenance (Gosling, 1982, 1990; Richardson, 1993).

The beaver (*Castor* spp.) defends a territory and usually lives in colonies consisting of an adult pair, kits, yearlings, and sometimes subadults (Bradt, 1938; Aleksiuk, 1968; Bergerud and Miller, 1977; Novak, 1977; Svendsen, 1980a, 1989; Nolet and Rosell, 1994). In a review of 13 earlier studies of the Eurasian beaver (*C. fiber*), Rosell and Parker (1995) found an average colony size of 3.8 individuals (SD = 1.0, range = 2.4–5.5). The beaver is monogamous, which is rare among Rodentia, and can occupy the same lodge for many years (Wilsson, 1971; Hodgdon and Lancia, 1983; Svendsen, 1980a, 1989).

Olfactory communication is likely to be important to beaver as they have poorly developed long-distance acoustic communication (with the exception of tail-slapping), are primarily nocturnal, and therefore are less reliant upon visual communication (e.g., Wilsson, 1971; Schulte, 1993). The beaver possesses two pairs of organs used in scent-marking (Hodgdon, 1978; Walro and Svendsen, 1982; Valeur, 1988). These are located in two cavities between the pelvis and the base of the tail and consist of two castor sacs and two anal glands. Castoreum from the castor sacs and/or oil from the anal glands are secreted onto small piles of mud and debris close to the water's edge (Wilson, 1971; Svendsen, 1980b; Rosell and Nolet, 1997), although castoreum is probably used more (Bollinger, 1980; Tang et al., 1993; Schulte et al., 1994). All age classes and male and female mark within the territory (Aleksiuk, 1968; Wilsson, 1971; Butler and Butler, 1979; Svendsen, 1980b; Buech, 1995).

One of the main functions of scent-marking appears to be the maintenance of territorial rights in both North American (*C. canadensis*) (Houlihan, 1989; Welsh and Müller-Schwarze, 1989), and Eurasian beaver (Rosell and Nolet, 1997). If the primary function of scent-marking were territorial defense, then marking is predicted to be most frequent when transient animals from other families are most likely to enter occupied areas, i.e., in spring or early summer

when dispersal of subadult beaver normally occurs (Beer, 1955; Bergerud and Miller, 1977; Molini et al., 1980; Svendsen, 1980a).

The central problem facing all animals that scent-mark their territories is where to place their marks. Given the constraints of time and energy, scent marks should not be deployed at random, but instead in an organized pattern that maximizes their chance of being discovered by the individuals to whom they are directed, and that gives the earliest possible warning to a potential trespasser. Such a place might be territorial borders (Gosling, 1982; Gorman, 1990), and the upstream edge of the territory should be most frequently marked if the movement of dispersing individuals is predominantly downstream. The pay-off to the owner is the reduced costs of competition (Gosling, 1986; Gosling and McKay, 1990).

In this paper we focus on the temporal and spatial distribution of scent-marking in the Eurasian beaver during an entire year to illustrate seasonal differences in the pattern of marking. We investigated the hypothesis that Eurasian beaver scent-mark as a means of territory defense. The following two predictions were tested: (1) the number of scent marks in territories is highest during spring when dispersion of subadults normally occurs; and (2) scent marks are not randomly placed inside the territory.

METHODS AND MATERIALS

Study Area. The study was conducted on a 9.2-km section of the Bø River in the municipality of Bø (59°25′N, 09°03′E), Telemark County, Norway. The part of the river studied averages 35 m in width and meanders through mixed woodland and agricultural countryside dominated by marine and fluvial deposits (Bergan, 1996). Vegetation along the river consists mainly of alder (*Alnus incana*) with lesser amounts of willow (*Salix* spp.), birch (*Betula pubescens*), aspen (*Populus tremula*), rowan (*Sorbus aucuparia*), Norwegian spruce (*Picea abies*), and Scots pine (*Pinus sylvestris*). During winter, a hydroelectric power station further upstream regulates flow, keeping the river ice-free. This provided us with the opportunity to study scent-marking behavior uninhibited by the usual constraints of winter ice. The river has been occupied by beaver since the 1930s (Olstad, 1937).

Study Animals and Colonies. The study was conducted between April 1, 1995, and March 31, 1996. The number of animals in each colony was determined by direct counts using light-sensitive binoculars. Counts were made from the riverbank or from a canoe around dawn and dusk, at approximately 14-day-intervals, both in August and September of 1995 and on many other occasions throughout the autumn. The animals were classified as kits, yearlings, and adults ≥ 2 years old based on body size and tail size (length $\times$ width) (Townsend,

1953; Patric and Webb, 1960; Jackson, 1991; Van Deelen, 1994). The sound of the tail-slap also gave a good indication of the age class of the animal, as smaller animals make lighter slap sounds.

Delineation of Territories. Territorial borders for each colony were determined from regular sight observations of animals moving up- and downstream of the lodge throughout the year. Individual movements were plotted on maps. Particular notice was made of the extreme distances moved away from the lodge in both directions. The borderline between territories was drawn at a point midway between the extremes of neighboring territory holders. The two colonies at the outskirts of the study area (colonies 1 and 7) were delineated naturally from colonies outside the study area by waterfalls or riffles, which also constituted territorial boundaries. The closest neighbor was found by measuring the midstream distance between the two occupied lodges. The territory size was defined as the total length of river bank (sum of both sides) between upper and lower territorial borders of a colony.

Scent-Marking. Each side of the river within the study area was searched from a canoe once every 14 days (biweekly) for newly used scent mounds from April 1, 1995, to March 31, 1996. Scent mounds are small piles, usually of mud and debris, scraped together by beaver upon which secretion from the castor sacs and/or anal glands are deposited (Aleksiuk, 1968; Wilsson, 1971). A freshly marked scent mound, i.e., with a scent detectable by the human nose at 2 cm or more, was termed a scent mark. This definition also included marks directly on the ground or on tussocks. The minimum distance between two different scent mounds was 10 cm. If a scent mound did not have a smell detectable by the human nose, it was thought to be old and excluded from analysis (see, however, Bollinger, 1980; Schulte, 1993). Each scent mark was labeled for recognition, either with a small wooden stake placed 0.5–1 m behind the mark, or by writing the number on natural objects such as trees. All scent marks found during each biweekly trip were registered on a 1:5000 map. After each land visit, boots were cleaned in water to minimize transport of scent from one area to another. Whenever possible, marks were smelled from the canoe, i.e., without actually stepping out.

The year was divided into four periods, which roughly coincided with four different behavioral events for the beaver: breeding (January–March) (Wilsson, 1971), dispersal of subadults (April–June) (e.g., Molini et al., 1980; Svendsen, 1980a), kits emerging from the lodge (July–September) (Wilsson, 1971), and winter preparations (October–December) (Wilsson, 1971).

Statistical Methods. Mean values are presented with standard errors. We used nonparametric statistics in accordance with Siegel and Castellan (1988). Nonparametric tests were corrected for ties. Probability values are two-tailed, and 5% was used as the level of significance. Mean values $\pm$ 2 SE give approximately 95% confidence intervals.

RESULTS

Seven colonies were located within the study area. A total of 2628 scent marks ($\bar{X}$ = 375 ± 85/colony/year, range 197–431) were found within the seven territories during the study. From November 30, 1995, to March 9, 1996, all scent marks were located on snow (N = 380). Twenty-eight beaver were recorded by sight observations during the intensive sight observation periods in August and September 1995. Colony size varied from three to seven individuals ($\bar{X}$ = 4 ± 0.6, N = 7). Seventy-one percent were adults, 22% were 1-year-olds, and 7% were kits. Only colony 7 produced young in 1995 (two kits were born). All colonies, except colony 4, had one yearling. The study area had a density of 0.76 colonies and 3.0 animals per kilometer of stream length. Average territory size was 2.6 ± 0.3 km (length of both banks, range 1.5–3.4). No seasonal difference in territory size was observed, and the borders were stable throughout the year.

Number of Scent Marks. No significant correlation was found between total number of scent marks of a colony and distance from its main lodge to the main lodge of the nearest neighboring colony (Spearman rank-correlation coefficient r_s = 0.095, N = 7, P = 0.840), or between the number of scent marks and territory size (r_s = 0.564, N = 7, P = 0.187). Beaver in colonies with two neighboring territories (N = 5) scent-marked on the average 373 ± 44 times, not significantly different from colonies (N = 2) with only one neighboring territory ($\bar{X}$ = 382 ± 49, Mann-Whitney U test, U = 4.0, P = 0.699).

Seasonal Variation. There was a significant difference in the mean number of scent marks per colony between each of the four seasons (Kruskal-Wallis one-way ANOVA, χ^2 = 21.04, df = 3, P = 0.001) (Figure 1). The mean number of scent marks per colony (N = 7) was higher during the dispersal of subadults ($\bar{X}$ = 199 ± 11), than during the other three periods (kits emerging from the lodge = 87 ± 15; winter preparations = 26 ± 5; breeding = 63 ± 10). Scent-marking was most frequent in May (weeks 18–21). A peak in the number of scent marks occurred in all colonies between the beginning of April (week 14/15) and the end of May (week 20/21). On June 21, 1995, at 14:15 hr, we observed a beaver (probably a subadult) on land inside the territory of colony 5. It entered the water, and we followed the animal at a distance by canoe as it moved downstream in a headwind. It swam rapidly past the lodge and through the territory of colony 6. When entering the border area between colony 6 and 7, it slowed down, and approached a site at the shoreline with a high density of scent marks, while simultaneously making up-and-down sniffing movements with the head. It then swam close to shore at a lower speed until reaching the lodge of colony 7, into which it dove. We assume that this was a dispersing subadult from colony 7.

Scent-marking almost ceased during weeks 22/23 when a flood raised water

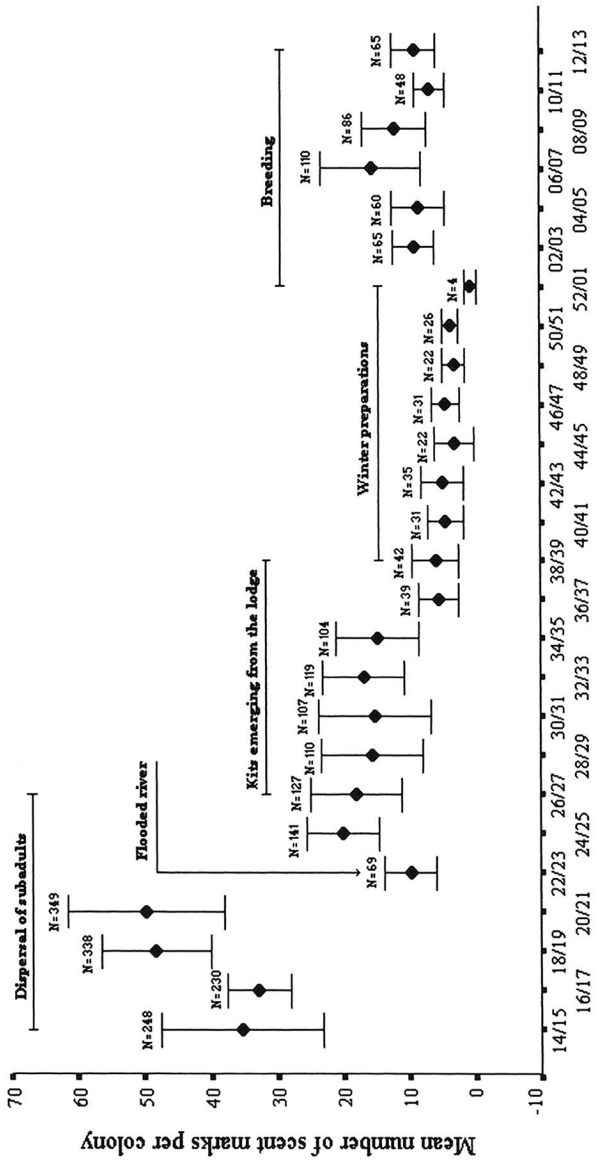


FIG. 1. Mean number of scent marks in seven adjacent territories along the Bø River observed at 14-day intervals throughout one year (April 1, 1995–March 31, 1996). Vertical bars show the 95% confidence intervals. *N* is the total number of scent marks for each 14-day period in all seven territories combined. A flood during weeks 22/23 hampered scent-marking activity.

levels by 90 cm. Scent-marking increased briefly thereafter, then gradually declined when kits emerged from the lodge and during winter preparations. The number of scent marks increased again in January–February (weeks 2–9) during breeding, and then decreased briefly, before increasing again during the dispersal of subadults.

Location of Scent Marks. The majority of scent marks were clumped near territorial borders within a zone of about 150 m throughout the year (Figure 2). During the winter preparation period, beaver scent-marked almost exclusively at territorial borders. Beaver in the two colonies at the peripheries of the study area (colonies 1 and 7) concentrated scent marks only along the territorial border adjacent to their single neighbor colony.

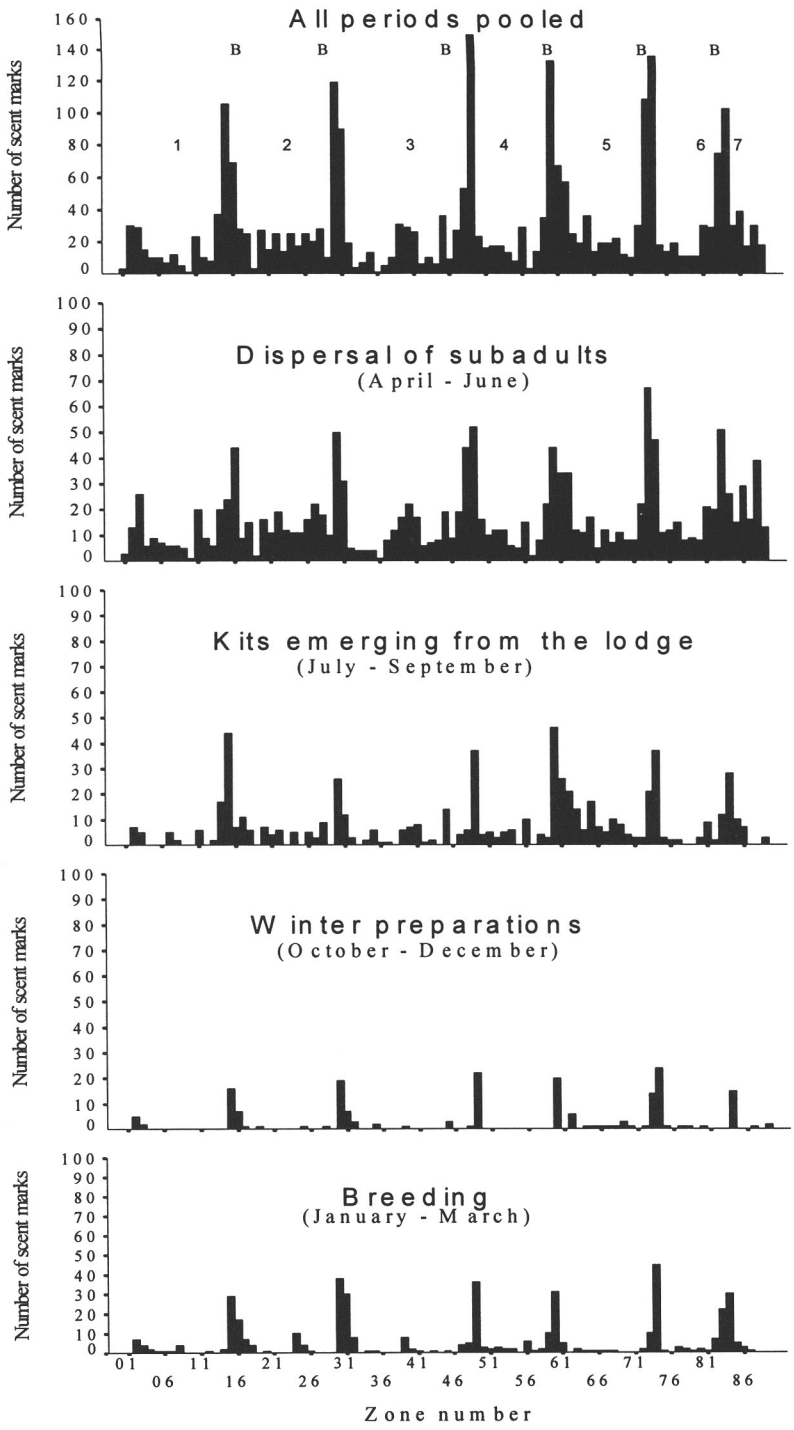
Significantly more scent marks were located between the lodge and the upstream territorial border ($\bar{X} = 267 \pm 46$, $N = 7$) than between the lodge and the downstream border ($\bar{X} = 107 \pm 35$, $N = 7$) (Mann-Whitney U test, $U = 6.0$, $P = 0.018$) (Figure 2). Beaver in all colonies, except 1 and 2, scent-marked significantly more upstream of the lodge than downstream. This was true, even when the nearest neighbor's lodge was located downstream (Figure 2).

DISCUSSION

Temporal Distribution. The number of scent marks was highest in spring (April–May). This is in accordance with our first prediction and in agreement with earlier studies for both species of beaver (Butler and Butler, 1979; Müller-Schwarze and Heckman, 1980; Svendsen, 1980b; Rosell and Nolet, 1997). The random observation of the apparently dispersing beaver from colony 7 was analogous to that described by Hodgdon (1978) for a beaver dispersing through an occupied territory. Fresh scent marks were indeed found in the immediate vicinity of the on-land observation of this beaver and may be the explanation for why colony 5 scent-marked more frequently during the spring than the other colonies.

The considerable and rapid increase in water level at the end of May 1995 probably led to a constant washing out of scent marks, and hence to a low number of recordings in that particular period. A similar phenomenon was observed in the Netherlands (Rosell and Nolet, unpublished).

Hodgdon (1978) suggested that the intense marking activity following ice-out might have been a delay of marking activity caused by the physical barrier of winter ice, and thus not a true reflection of actual marking cycles. He suggested that an analysis of resident family scent-marking cycles at more southerly latitudes, where the ice barrier was absent, should provide an assessment of any relationship between gonadal activity and marking. Indeed, despite its northerly


All periods pooled
B
1
2
3
4
5
6
7
Dispersal of subadults
(April - June)
Kits emerging from the lodge
(July - September)
Winter preparations
(October - December)
Breeding
(January - March)
Number of scent marks
01
06
11
16
21
26
31
36
41
46
51
56
61
66
71
76
81
86
Zone number

location, the Bø River remains ice-free throughout the year due to hydroelectric regulation. Results from our study therefore suggest that the high frequency of scent marking in spring probably is primarily associated with a peak in dispersal of subadults at this time and is not a delay in the scent-marking peak due to winter ice. However, prolonged low temperatures and ice can limit beaver activity (Wilsson, 1971; Lancia et al., 1982; Hodgdon and Lancia, 1983). Although initial natal dispersal appears to occur most commonly in spring (Molini et al., 1980; Svendsen, 1980a), it may occur at other times of the year. Van Deelen and Pletscher (1996) found that the dispersal date was highly variable, ranging from April 7 to August 20 (mean: May 17), as was settlement date (mean: July 24; range: April 9–November 12). Hodgdon (1978) found that dispersal took place from March to September. Hartman (1994) found that three of nine beaver in his study dispersed as yearlings in early fall, and one in early winter. If waters are icebound, dispersal will be impossible until ice break-up. However, if freeze-up does not occur, beaver may be highly mobile throughout the year (Payne, 1984). Exploratory movements outside the natal territory have been observed in several studies (Wilsson, 1971; Payne, 1982; Van Deelen, 1991; Hartman, 1997), and presumably can develop into true dispersal. A substantial drop in temperature and an eventual freeze-up might, on the other hand, drive some explorers back to the natal colony. The continually ice-free state of the Bø River allows dispersion throughout the entire year. However, low water temperatures make prolonged swimming a very costly activity (Nolet and Rosell, 1994).

Spatial Distribution. If the primary function of beaver scent-marking was territory defense, then markings might be expected to cluster near territorial boundaries. Hediger (1949) commented that many species deposit scent where they meet or expect rivals; e.g., near territory borders. Peters and Mech (1975) reported that wolves (*Canis lupus*) concentrated scent marks at the periphery of the territory. The same pattern was also found in Chinese water deer (*Hydropotes inermis*) (Sun et al., 1994), in aardwolves (*Proteles cristatus*) (Richardson, 1991), in tigers (*Panthera tigris*) (Smith et al., 1989), and in badger (*Meles meles*) (Kruuk, 1978; Kruuk et al., 1984). Aleksiuk (1968) found most marking points for the North American beaver at the edge of territories, but some also were located near the lodge. Richard (1967) suggested that the Eurasian beaver marked most frequently near territorial boundaries. Rosell and Nolet (1997) also found a higher number of scent marks near the borders. Nitsche (1985a,b), in

FIG. 2. Total number of scent marks in each 100-m zone during each of the four three-month periods and for all periods pooled for seven adjacent territories along the Bø River during one year (April 1, 1995–March 31, 1996). "B" indicates the location of borders between territories based on the movement patterns of colony members. Numbers indicate the approximate position of the lodge in territories 1–7. Zone 1 is positioned furthest upstream.

contrast, found that the central part of a territory was more intensively marked than the peripheries (Eurasian beaver).

In this study, scent marks were clumped near territorial borders, which is in accordance with our second prediction. In this manner, intruding beaver, upon entering a foreign territory, quickly discover that the area is already occupied. This general pattern was maintained throughout the year. From October to December, when marking activity was minimal, almost all marking occurred at territorial borders. In this manner, beaver presumably maximize the effect of the scent-marking process at a time of the year when time and energy are mainly allocated to preparation for winter.

More scent marks were located upstream than downstream of the lodge, which is also in accordance with our second prediction. This was the case regardless of the location (up- or downstream) of the nearest neighbor. Colonies 1 and 2 were exceptions. Colony 1 had no close neighbors upstream, and therefore presumably had less reason to scent-mark intensively here. As for colony 2, we have no explanation for why it deviated in this respect. In contrast, Müller-Schwarze (1992) found no difference in the frequency of upstream and downstream marking, and concluded that if scent-marking provides information by water-borne chemicals, it is not reflected in the number of scent mounds built by downstream beaver.

Whether marking activity is concentrated up- or downstream of the lodge may be dependent upon the predominating direction of dispersal in a particular watershed. Downstream dispersal would presumably be the most energy efficient, in which case concentrating most scent marks at the upstream border would be the most effective means of informing potential intruders. We do not know the main direction of dispersal in our study area, although beaver have been shown to disperse both upstream and downstream (Leege, 1968; Van Deelen and Pletscher, 1996). Another explanation for a predominance of upstream marking would be that intruders entering from a downstream direction automatically receive an almost continual flow of chemical scent information in the surface film from all upstream territories. Thus the water segment of a beaver's territory presumably is readily covered in this manner. Indeed, swimming beaver keep their nostrils at the water level, thus enabling them to sense chemical messages from neighboring beaver concentrated within the surface film (Grønneberg and Lie, 1984).

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EFFECTS OF PLANT IDENTITY AND CHEMICAL CONSTITUENTS ON THE EFFICACY OF A BACULOVIRUS AGAINST *Heliothis virescens*

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Abstract—Baculoviruses are arthropod-specific, dsDNA viruses primarily used to control lepidopteran pests. A limitation of the use of baculoviruses for pest control is that their efficacy is modifiable by host-plant chemicals. The levels of phenolic substrates and two foliar oxidative enzymes, peroxidase (POD) and polyphenol oxidase (PTO), were significant predictors of disease caused by a baculovirus in *Heliothis virescens* fed on either cotton or lettuce; POD was the more influential of the two enzymes. The higher the plant phenolase activity, the lower the percent mortality and the slower the insects died from viral infection. Whether a particular class of phenolic substrates was correlated with enhanced or attenuated baculoviral disease depended upon context, i.e., admixture. Diminution of viral efficacy by plant oxidative activity may compromise the compatibility of baculoviruses with other components of an integrated pest management system such as host plant resistance.

Key Words—Baculovirus, host-plant resistance, peroxidase, polyphenol oxidase, *Heliothis virescens*, tritrophic interactions, cotton, lettuce, phenolics.

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INTRODUCTION

There is increasing interest in the use of microbial control agents such as the nuclear polyhedrosis viruses (NPVs, a subgroup of the Baculoviridae) for the control of several important agricultural pests, particularly some in the family Noctuidae (e.g., *Heliothis*, *Helicoverpa*, and *Spodoptera* spp.). This renewed interest is due in part to the safety of baculoviruses to nontarget organisms (Heinz et al., 1995; McCutchen et al., 1996) and the ability of researchers to engineer baculoviruses to express gene products, resulting in improved speed of kill (Bonning and Hammock, 1992, 1996; Miller, 1995). A limitation of the use of baculoviruses as biopesticides is that their efficacy, as well as that of a number of entomopathogens, is unpredicatably modified by the host plant (see Duffey et al., 1995, for review; Farrar et al., 1996). Previous studies have shown that when insects were fed foliage from different plant species (Keating and Yendol, 1987; Keating et al., 1990; Forschler et al., 1992) or plants of disparate quality (Ebihara, 1966; Hayashiya et al., 1968; Sosa-Gomez et al., 1991; Hunter and Schultz, 1993) treated with baculoviruses, susceptibility to lethal infection[6] varied as much as 50-fold. In some cases, strong correlations (the sign dependent on the chemical) have been shown between foliar levels of certain phytochemicals and viral efficacy (Uchida et al., 1984; Felton et al., 1987; Keating et al., 1988, 1989; Felton and Duffey, 1990; Young et al., 1995).

Most investigations of the impact of host plant on baculoviral disease in insects have been phenomenological. Although it is well established that plant type and plant chemistry often have a profound influence on the course and severity of baculoviral disease (reviewed by Duffey et al., 1995), identification of the causal agents and elucidation of their mechanisms of action have been ill-defined or neglected (see Hayashiya et al., 1976; Keating et al., 1988, 1990; Felton and Duffey, 1990; Watanabe et al., 1990 for notable exceptions; and review by Duffey et al., 1995), primarily because of the experimental difficulty of establishing causality and mechanism. Several groups are making notable progress in overcoming some of these difficulties by the study of the impact of phenolics upon baculoviral disease in larval hosts (Felton et al., 1987; Felton and Duffey, 1990; Keating et al., 1990; Hunter and Schultz, 1993; Young et al., 1995). In a few cases the mechanism of inhibition of viral disease is being unraveled (Hayashiya et al., 1976; Uchida et al., 1984; Felton and Duffey, 1990; Keating et al., 1990; Watanabe et al., 1990). Several critical concepts emerge from these studies that bear on the above difficulties:

[6]We use the term "lethal infection" rather than simply "infection" because we have no evidence that plant compounds prevent binding and/or assimilation of the nucleocapsids after release from the polyhedral occlusion body. Infection may still occur in the presence of inhibitory plant compounds, but the infected midgut cells may be damaged and sloughed as a result of oxidative stress before the virus can spread beyond the midgut, effectively eliminating the infection from the insect.

1. Multiplicity: viral disease is simultaneously influenced by more than one phytochemical, each exerting its effect simultaneously. Thus, the influence of phytochemicals upon viral disease is multivariate; hence, there are causes and effects, not a cause and an effect.

2. Interactivity: a chemical does not influence viral disease independently of other phytochemicals. It interacts with other dietary components, often in nonlinear fashions; hence, context (what other chemicals are in admixture) is important.

3. Multifunctionality: a chemical often exhibits multiple mechanisms of action in its interactions with other chemicals and/or with baculoviruses, the degree and number being dependent upon context.

Our study was designed to reinforce the importance of the above concepts in understanding the impact of plant phenolics in crop plants upon the progression and severity of baculoviral disease in noctuid larvae. Phenolics do not act in isolation when ingested by the insect; they are modified by pH, redox conditions, oxygen availability, and/or other dietary chemicals (Appel and Schultz, 1992; McEvily et al., 1992; Appel, 1993, 1994; Duffey and Stout, 1996; Johnson and Felton, 1996). The plant oxidative enzymes polyphenol oxidase (PPO) and peroxidase (POD) act upon a variety of phenolics, particularly *o*-dihydroxyphenolics (catecholic phenolics), to produce highly reactive quinones (Pierpoint, 1983) and free radicals (Butt, 1981; Butt and Lamb, 1981; Robinson, 1991). Reactive products from phenolic oxidation can bind to proteins, damage membranes, and are implicated as defensive responses of plants against herbivores (Felton et al., 1989, 1992; Appel and Schultz, 1992; Bi et al., 1994; Summers and Felton, 1994; Duffey and Stout, 1996; Stout and Duffey, 1996). More recent reports implicate the oxidation of phenolics by oxidative enzymes as important processes capable of inhibiting baculoviral disease (Felton and Duffey, 1990).

In addition to catecholic phenolics, polymeric phenolics (tannins) have been implicated as factors capable of inhibiting mortality of susceptible hosts treated with baculoviruses (Keating et al., 1988, 1989, 1990; Young et al., 1995). Tannins in oak and cotton foliage were negatively correlated with larval mortality caused by baculoviruses of *Lymantria dispar* (Keating et al., 1988, 1990) and *H. zea* (Forschler et al., 1992), respectively. These reports might lead one to conclude that tannins are the causal agents of inactivation of baculoviruses on cotton. However, this conclusion is not fully supported by the evidence. Condensed tannins are not sufficiently correlated with decreased mortality by baculovirus in vivo (Keating et al., 1990), although negative relationships between condensed tannins and viral disease have been demonstrated by diet incorporation (Keating et al., 1989; Young et al., 1995). Finally, treatment of *H. zea* larvae on cotton cultivars that are isogenic except for tannin content (a high tannin line vs. a low tannin line) did not differ in susceptibility to bacu-

loviral infection (G. W. Felton and S. Y. Young, personal communication). Thus, poor performance of baculoviruses as pest control agents on cotton requires further exploration.

Herein, we test the effects of POD, PPO, and various classes of phenolics on the virulence of baculoviruses. These results suggest that oxidative processes catalyzed by peroxidase occurring in the insect midgut are, at least in part, responsible for the observed attenuation of viral disease on certain host plants. In this study, we also demonstrate an important paradigm concerning viral efficacy—that phytochemicals influence viral disease in a manner that is: (1) multiplex, (2) interactive, and (3) multifunctional, each of which is dependent on chemical context.

METHODS AND MATERIALS

To evaluate our hypothesis that plant phenolase activity is a major factor responsible for inhibiting baculoviral disease, we identified and quantified several plant chemical factors (e.g., phenolics, protein, and the oxidative enzymes PPO and POD), which we correlated by multiple regression with viral efficacy as modified by four different host plants of *H. virescens* with a permissive virus, *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV). Specifically, we performed two sets of bioassays in which we examined two different response variables (percent mortality and rate of mortality) as functions of level of plant protein, phenolics, and oxidative enzyme activities. In bioassay 1, percent mortality (modeled by logistic regression) of third-instar *H. virescens* dosed with wild-type AcMNPV (WT AcMNPV) at a range of viral doses was determined. In bioassay 2, the rate of mortality (modeled by fitting survivorship curves) of neonate larvae of *H. virescens* infected with an LD_{99} was determined. Having determined from bioassay 1 above that host plant has only a minor influence on percent mortality at the LD_{99}, we chose to investigate whether host plant exerts any influence at this high viral dose on rate of mortality. Thus, larvae were dosed at an LD_{99} with either WT AcMNPV or a recombinant virus derived from AcMNPV, termed AcAaIT, which expresses an insect-selective neurotoxin derived from the scorpion *Androctonus australis* (McCutchen et al., 1991). AcAaIT kills larvae of *H. virescens* approximately 30% faster than wild-type virus (McCutchen et al., 1991; Hoover et al., 1995).

For these two sets of bioassays, the following methods were used:

Plants. Two cultivars of cotton (*Gossypium hirsutum*, cv. Acala SJ2 and Delta pine 51) and lettuce [*Latuca sativa* L., cv. Valmaine (romaine) and Salinas (iceberg)] were grown in a greenhouse under natural conditions to the four to five-leaf stage. These plants were chosen because these two species differ in

phenolic, oxidative enzyme, and/or protein levels. To obtain an array of phenolic and enzyme levels within a plant species for our regressions, which evaluated the influence of phytochemicals on rate of mortality (bioassay 2), we did not attempt to regulate photoperiod or temperature (except for the maximum temperature) in the greenhouse. Thus, we did not use artificial lighting; the photoperiod averaged 10L:14D in winter and 16L:8D in summer. The minimum/maximum temperatures averaged 21/27° in winter and 24/30°C in summer. Bioassay 1 above was performed in the summer months.

Insects. Tobacco budworm eggs were obtained from the United States Department of Agriculture Agricultural Research Station (Stoneville, Mississippi). Neonate larvae reared to third instar were placed individually on 8 cm^3 cubes of semisynthetic diet (BioServ, Inc.) in 24-well tissue culture plates (Fisher, St. Louis, Missouri) and maintained at 26 ± 1°C and 16L:8D.

Viruses. WT AcMNPV (C6 clone) (Ayers et al., 1994) and the recombinant AcAaIT were amplified in larvae of *H. virescens* and were extracted, partially purified, and stored until use as described in Hoover et al. (1995).

Foliar Chemical Assays. The protein content was determined by extraction of foliage in 0.5 N NaOH followed by the Bradford assay (Bradford, 1976), with ribulose, 1, 5-diphosphate carboxylase–oxygenase as a standard and the addition of 3% polyvinylpyrrolidone to the assay mixtures to minimize interference from phenolics (Jones et al., 1989). PPO and POD activities were determined colorimetrically with caffeic acid and guaiacol/H_2O_2, respectively, as substrates. Activities were measured as the increase in OD_{470} per minute per gram (Ryan et al., 1982; Felton et al., 1989). Catecholic phenolic content was determined colorimetrically with a 0.5% diphenylborinic acid–ethanolamine complex. Chlorogenic acid and rutin were used as standards at OD_{390} and OD_{440}, respectively (Broadway et al., 1986). Total phenolics were measured with the Folin-Ciocalteau reagent with chlorogenic acid as a standard (Singleton and Rossi, 1965). We calculated noncatecholic phenolics as the difference between total phenolics and catecholic phenolics. Total gossypol content was measured by the aniline method (Hedin et al., 1992) and reported as gossypol equivalents with gossypol acetate as a standard. Because this method also measures other aromatic terpenoid aldehydes (except benzaldehyde), HPLC was used to determine the terpenoid aldehyde content of the two cotton cultivars (courtesy of R. D. Stipanovic, USDA, ARS; Stipanovic et al., 1988). Condensed tannins were determined by the butanol/HCl assay (Lane and Schuster, 1981) using purified condensed tannins extracted from Acala cotton as a standard (Hagerman and Butler, 1980).

Percent Mortality of Third-Instar H. virescens *Dosed with AcMNPV.* Leaf disks, 0.5 cm diameter, were cut from foliage of each plant (12–15 plants of each cultivar per replicate) and placed on agar in 24-well tissue culture plates. Treatments consisted of plant cultivar and viral dose. Four to five leaf disks

from each of the 12 plants of the same cultivar were distributed among all viral treatments (four viral doses per plant type). Viral inoculum suspended in double-distilled H_2O was applied to each leaf disk at 3000, 300, 100, or 30 polyhedra/larva representing the approximate LD_{99}, LD_{75}, LD_{50}, and LD_{25}, respectively. Viral inoculum was allowed to dry at ambient temperature. LDs were determined by dosing a group of insects on artificial diet as controls concurrently with each replicate.

Within 6 hr after molting, third instars were removed from diet and starved overnight to void gut contents. Starved larvae were transferred individually to a leaf disk. Insects that consumed their entire virally tainted leaf disk or diet cube were transferred individually to excess artificial diet in 35-ml cups and maintained until death or pupation at 26 $\pm$ 1°C and 16L:8D. Mortality was scored at eight to nine days after infection. There were 35–48 larvae per dose per treatment, and the experiment was replicated three times. Foliar chemical content was determined on the same day that leaf disks were prepared.

Data were analyzed by logistic regression to determine if the probability of an insect dying could be predicted from chemical content (Statistica, StatSoft; Kalbfleisch and Prentice, 1980; Collett, 1994). The model estimates the unknown parameter coefficients as follows: $\log (p/1 - p) = \beta X$, where $\beta X = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots$. Thus, the probability of dying $(p) = \exp \{(\beta X)/[1 + \exp (BX)]\}$. Parameter coefficients β with a positive sign indicate a variable that increases the probability of an insect dying; negative coefficients indicate a decrease in the probability of dying.

We used two separate models to determine if the probability of an insect dying could be predicted from phytochemical levels (independent variables). For the first model, we analyzed pooled data with plant type as a categorical variable. For the second model, we analyzed each plant species separately (i.e., the two cotton cultivars were analyzed separately from the two lettuce cultivars). Analysis of the pooled data permitted us to ask if plant type is a critical variable, i.e., can viral performance be predicted by simply knowing plant type, or does prediction of viral efficacy require knowledge of plant chemistry among plant types? Analysis of the two plant species separately allowed us to ask if the same phytochemical variables influence viral efficacy in the same way in the two different plant species. Parameter coefficients for plant type were also used to calculate odds ratios as described in Armitage and Berry (1994). Each independent variable was regressed separately in combination with viral dose, followed by backward stepwise logistic regression to determine the most predictive model. In addition, we subjected the arcsin-transformed percentage mortalities for each plant cultivar at each viral dose to two-way ANOVA to further test whether viral performance can be predicted by simply knowing plant type (Steel and Torrie, 1980).

Rate of Mortality of Neonates Infected with WT AcMNPV or Recombinant AcAaIT. Time–response bioassays were conducted to determine the influence of host plant on the rate of mortality of neonate *H. virescens* treated with baculoviruses. True leaves were removed from plants and placed individually in 100- × 15-cm Petri dishes on top of a 9-cm piece of Whatman No. 1 filter paper moistened with 1 ml of water. Leaves were exchanged for fresh ones every 48 hr. Two leaves were used from each plant per treatment group. Neonate larvae of *H. virescens* were droplet fed a viral suspension of 2000 polyhedra/ μl (LD_{99}) of either AcAaIT or WT AcMNPV (Hughes et al., 1986). The viral formulation contained polyhedra in double distilled H_2O with 5% blue food dye (v/v) and 6% maltose (w/v). Controls consisted of larvae fed the same formulation without virus. Two hours after dosing, larvae that had consumed the viral suspension (or H_2O) were transferred individually to a leaf, and the Petri dish was sealed with parafilm. Larvae were maintained at 26 $\pm$ 1°C and 16L:8D, and mortality was scored every 4–8 hr, depending upon the mortality rate. Thirty-five larvae were infected per bioassay for each treatment. Bioassays were replicated two to three times during each of the four seasons of the year for a total of nine replicates. Each replicate also contained a group of insects fed on artificial diet instead of foliage as a control to determine if monthly differences in rate of mortality were the result of seasonal variability in physiological susceptibility of the insects.

Survival data were analyzed by Cox's proportional hazards model (S-plus; Kalbfleisch and Prentice, 1980; Collett, 1994) to determine if the survival curves could be predicted from levels of phytochemicals (independent variables). In addition, plant type and month were entered as categorical variables. The model calculates the baseline hazard function $\lambda(t) = \lambda_0(t) \exp(\beta X)$ where $\lambda_0(t)$ is the baseline hazard function representing the differential probability of dying at time (t), given survival to time (t). The βs are unknown parameters to be estimated by the model and Xs are the levels for each independent variable. By definition $\exp(\beta X) = \exp(\beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots)$ and is the amount by which $\lambda_0(t)$ is multiplied. Thus, an estimated parameter coefficient with a positive sign indicates a variable correlated with increased speed of kill; a coefficient with a negative sign indicates a variable correlated with decreased speed of kill. The best model was chosen by comparing the likelihood ratio chi-square (LRCS) values for each model.

We entered each variable in the model separately, followed by backward stepwise multiple regression to determine the most predictive model. We fitted two types of models. For the same reasons as described in the methods for the first set of percent mortality bioassays, we first examined the influence of phytochemicals on the survival curves using pooled data; we then evaluated models separately for each plant species. Because survival times were known only up

to an interval of time, the survival times of the insects were estimated as the midpoints of the intervals in which they died. The Kaplan-Meier product limit estimator was used to estimate the LT_{50}s for each treatment (S-plus; Kalbfleisch and Prentice, 1980; Collett, 1994).

RESULTS

Percent Mortality of Third-Instar H. virescens *Dosed with AcMNPV*

Combined Cotton and Lettuce Model. There was a dramatic influence of host plant on lethal infections in *H. virescens* treated with baculovirus, with the greatest impact occurring at lower doses (Figure 1). Except at the highest viral dose, mortality appeared highest on iceberg lettuce, lower on romaine lettuce, and lowest equally on the two cultivars of cotton; however, these differences were not significant at the 5% level using ANOVA to compare mean percent mortalities ($F = 1.62$, $df = 3, 9$, $P = 0.2030$). The odds of dying (odds ratios)

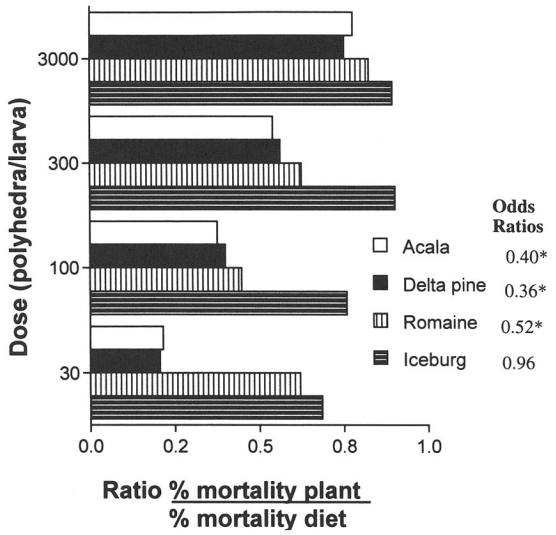

FIG. 1. Influence of host plant on mortality of third-instar *H. virescens* treated with AcMNPV. Bars represent the ratio of mean percent mortality for insects dosed on one of four host plants to mean percent mortality for insects dosed on artificial diet (control). Means are percentage mortality of three replicates (N = 12–15 plants/replicate). Odds ratios significantly less than 1 at the 5% level of significance (followed by asterisks) indicate treatments that protected the insect from dying compared to being dosed on artificial diet.

when insects were dosed on cotton or romaine lettuce were, however, significantly lower than the odds of dying on artificial diet. The odds ratios calculated for each plant type permitted us to determine whether any of the host plants protected the insect from lethal infection compared to the artificial diet control (Figure 1). The two cultivars of cotton were equally most protective, followed by romaine; iceberg lettuce was not significantly different from diet as a substrate for infection.

The lack of significant differences in mean percent mortality among host plants did not mean that viral efficacy was not influenced by host plant. On the contrary, larval mortality varied considerably within plant type from replicate to replicate as a function of plant phenolase activity, making it difficult to detect significant differences among treatments with ANOVA (a linear model which assumes a normal distribution). PPO activity was not detectable in cotton despite use of a diversity of substrates (chlorogenic acid, rutin, (±)-catechin, quercetin, and 2, 3-dihydroxybenzoic acid). Phytochemical levels within plant type varied considerably with photoperiod and temperature (experiments were replicated in late winter and early spring, data not shown), although within each replicate, cotton had significantly higher POD activity and phenolic content (catecholic and total phenolics) than lettuce (Table 1). Romaine lettuce contained slightly higher levels of all chemicals assayed than iceberg lettuce. The best relationship between mortality and an individual phytochemical variable was with POD (simple effect). With the exception of gossypol, only POD was useful for predicting mortality when other phytochemical variables were excluded from the model (Figure 2, simple effect). Total phenolics ($t = 1.54$, $P = 0.1270$), condensed tannins ($t = 0.80$, $P = 0.4237$), protein ($t = -0.21$, $P = 0.8367$), and plant type ($t = 1.61$, $P = 0.1109$) were not useful predictors for mortality either by themselves or in combination with other phytochemical variables.

When the best combination of phytochemical variables was examined, both POD and PPO had significantly negative effects on mortality (Table 2). Thus, the higher the level of these oxidative enzymes, the less likely the insect was to die from infection. In contrast, the higher the levels of catecholic phenolics and viral dose, the higher the probability of an insect dying. Finally, POD and PPO interacted in their effect on mortality ($\beta = +0.31$, Table 2). Thus, for a given level of PPO, the higher the level of POD, the less impact PPO had on decreasing the probability of an insect dying (a positive value for the interaction term added to the negative values of the individual terms). Similarly, the higher the PPO activity, the less impact POD had on protecting the insect from disease. However, POD was more influential in decreasing infectivity of the virus than PPO ($\beta = -1.19$ for POD vs. $\beta = -1.05$ for PPO; Table 2).

Separate Cotton and Lettuce Models. When data for the two cotton cultivars were analyzed separately from lettuce to permit examination of the influence of plant chemical content on mortality within a host plant species, the two models

TABLE 1. MEAN PHYTOCHEMICAL CONTENT OF FOUR HOST PLANTS USED TO TEST LETHAL INFECTIVITY OF AcMNPV TO *H. virescens*

Plant	POD (Δ Abs/g/min)	PPO (Δ Abs/g/min)	*o*-Dihydroxy phenolics (μmol/g)	Total phenolics (μmol/g)	Condensed tannins (μmol/g) (dry wt)	Gossypol (% gossypol equivalents) (dry wt)[a]	Protein (% Rubisco equivalents)
Acala cotton	170 ± 48	ND	17 ± 5.7	60 ± 22	26 ± 8.4	3.1 ± 0.80	2.7 ± 0.22
Delta pine cotton	184 ± 26	ND	14 ± 5.7	32 ± 14	23 ± 7.1	3.6 ± 0.49	3.0 ± 0.38
Romaine lettuce	15 ± 2.5	14 ± 1.4	4.0 ± 1.2	4.9 ± 0.81	NA	NA	1.5 ± 0.27
Iceberg lettuce	12 ± 1.6	11 ± 2.5	3.8 ± 0.90	4.3 ± 1.0	NA	NA	1.3 ± 0.29

Cotton cultivar	Hemigossypol	Gossypol	Heliocide H4	Heliocide H1	Heliocide H3	Heliocide H2
Acala SJ-2	0.49 ± 0.03	0.13 ± 0.01	0.13 ± 0.01	0.71 ± 0.02	0.39 ± 0.01	1.1 ± 0.04
Delta Pine 15	1.0 ± 0.03	0.40 ± 0.02	0.24 ± 0.01	0.72 ± 0.02	0.33 ± 0.01	0.90 ± 0.02

[a] Assay for gossypol also detects other aromatic terpenoid aldehydes. The concentrations of specific terpenoids (in μg/mg leaf tissue) are shown above. Concentrations were determined by HPLC using the appropriate standards (Stipanovic et al., 1988). NA = not applicable. ND = not detectable.

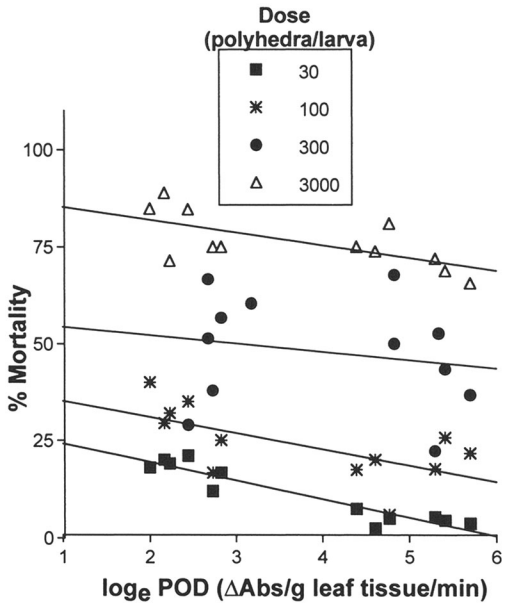

FIG. 2. Effect of peroxidase (POD) level on percent mortality of third-instar *H. virescens* dosed on one of four different host plants at 4 different viral doses. Data were analyzed for cotton and lettuce cultivars combined by logistic regression. The simplest model that fit the data consisted of viral dose ($\beta = +0.05$, $t = 19.54$, $P < 0.0001$) and $\log_e$ POD ($\beta = -0.10$, $t = -2.35$, $P = 0.0208$); Model chi-square = 525, $df = 2$, $P < 0.0001$; see Table 2 for best full model.

TABLE 2. COMBINED MODEL DEPICTING INFLUENCE OF PHYTOCHEMICAL CONTENT ON MORTALITY OF *H. virescens* TREATED WITH ACMNPV ON ALL PLANTS[a]

Variable	Parameter coefficient	*t* statistic	*df*	*P*
Catechols	+0.06	+4.65	88	<0.0001
PPO	−1.05	−4.82	88	<0.0001
POD	−1.19	−5.87	88	<0.0001
Viral Dose	+0.76	+19.74	88	<0.0001
PPO * POD	+0.31	+3.47	88	0.0008

[a]Model chi square = 555, $df = 5$, $P < 0.0001$; model equation: probability of dying (p) = exp (βX)/[1 + exp (βX)], where βX = [−0.74 + 0.06(Catecholic phenolics) − 1.19($\log_e$ POD) − 1.05(sqrtPPO) + 0.76($\log_e$ Viral dose) + 0.31($\log_e$ POD) * sqrtPPO]. Plant type was not significant ($t = 1.41$, $df = 88$, $P = 0.1618$).

were not the same (Table 3, simple model). Mortality on cotton and lettuce varied with POD and PPO activities, respectively. On cotton, the higher the POD activity, the lower the mortality. On lettuce, the higher the PPO activity, the lower the mortality.

The impact of condensed tannins on larval mortality depended upon whether it was regressed separately (with viral dose) or in combination with other phytochemicals (Table 3). By itself, the higher the condensed tannin content of cotton foliage, the lower the mortality (simple effect, negative coefficient). In combination with POD, however, tannins increased the probability of an insect dying from disease (full model, positive coefficient). POD and viral dose interacted in their effect on mortality (Table 3). Given that dose has a positive coefficient and POD a negative one, the higher the dose, the less negative was the slope of POD's impact on mortality. At higher viral doses, POD had less ability to inhibit viral disease. At lower doses, the influence of POD on inhibiting viral disease was much greater (more negative slope).

When insects were treated with baculovirus on lettuce, PPO, but not POD, was predictive of a lower probability of dying (Table 3, full model). In addition, insects dosed on lettuce were less likely to die from viral infection the higher the noncatecholic phenolic level of foliage (negative coefficient), but the probability of dying was increased by higher catecholic phenolic content of foliage (positive coefficient). Phytochemical effects in lettuce did not change whether the impact of the variables on mortality were considered separately or together.

Rate of Mortality of Neonates Infected with WT AcMNPV or Recombinant AcAaIT

Combined Cotton and Lettuce Model. A simplified examination of monthly survival times is depicted as mean LT_{50}s for each virus among the four plant types (Figure 3). LT_{50}s for insects fed on plants and artificial diet controls were slower in the summer months than the winter months. Mean foliar catecholic phenolic content for the four plant types followed a similar trend; catecholic phenolic levels were higher in the summer months and lower in the winter months (Figure 3). Although LT_{50}s for insects fed on plants parallel catecholic phenolic levels, the LT_{50}s on plants also paralleled monthly variability in LT_{50}s for insects fed on control diet. Thus, it is likely that seasonal differences in physiological susceptibility to viral infection also played a role in influencing lethal times. However, choosing one time point (the LT_{50}) in a survival curve (Figure 3) did not accurately reflect the statistical significance of month as a predictor of the entire survival curve, which is the probability of surviving up to time (t) for diet controls. Furthermore, month by itself was not the only significant predictor of the survival curves.

We examined the influence of host-plant chemical content as an indepen-

TABLE 3. SEPARATE MODELS DEPICTING INFLUENCE OF PHYTOCHEMICAL CONTENT ON MORTALITY OF *H. virescens* TREATED WITH AcMNPV ON COTTON OR LETTUCE[a]

Cotton				Lettuce			
Variable	Parameter coeff.	*t* statistic[b]	*P*	Variable	Parameter coeff.	*t* statistic[b]	*P*
Each variable regressed separately							
POD	−0.13	−0.69	0.4960	PPO	−0.06	−3.08	0.0035
Protein	−0.31	−2.46	0.0179	Protein	−0.01	−0.06	0.9527
Catechols	+0.01	+1.33	0.1912	Catechols	+0.07	+1.75	0.0870
Total phenolics	+0.06	+2.17	0.0354	Total phenolics	−0.18	−2.38	0.0214
Noncatechols[c]	+0.05	+2.16	0.0344	Noncatechols[c]	−0.32	−2.38	0.0214
Tannins	−0.01	−2.18	0.0346	POD	+0.11	0.44	0.6638
Gossypol	+0.03	+0.40	0.6946				
			Best full models				
Model[d]							
POD	−1.80	−2.46	0.0180	PPO	−0.05	−2.53	0.0150
Tannins	+0.02	+3.05	0.0039	Catechols	+0.98	+2.23	0.0309
Viral dose	+0.09	+5.92	<0.0001	Noncatechols[c]	−1.27	−2.21	0.0326
POD * dose	+1.13	+2.90	0.0058	Viral Dose	+0.05	+13.40	<0.0001
Model 2[e]							
Viral Dose	+0.05	+13.89	<0.0001				
POD	−0.75	−2.52	0.0156				
Catechols	+0.03	+2.75	0.0086				

[a] Data for cotton and lettuce were analyzed separately by logistic regression. Regressions were first run using viral dose + individual chemical variables (viral dose invariably had a P value of <0.0001 in combination with each chemical variable), followed by stepwise regression to determine the best full model for each plant species. For each model, the probability of dying (p) = $\exp(\beta X)/[1 + \exp(\beta X)]$. Cotton model 1: $\beta X = [-1.80(\log_e \text{POD}) + 0.02(\text{Tannin}) + 0.09(\text{viral dose}) + 1.13(\log_e \text{POD} * \text{Viral dose})]$; chi-square = 288, $df = 4$, $P < 0.0001$; model 2: $\beta X = [0.05(\text{viral dose}) - 0.75(\log_e \text{POD}) + 0.03(\text{catechols})]$; chi-square = 282, $df = 3$, $P < 0.0001$. Lettuce model: $\beta X = [-0.05(\text{PPO}) + 0.98(\text{Catechols}) - 1.27(\text{Noncatechols})]$; chi-square = 263, $df = 4$, $P < 0.0001$.

[b] $df = 45$ for all variables regressed separately with viral dose.

[c] Noncatechols = total phenolics − catecholic phenolics.

[d] $df = 41$.

[e] $df = 42$.

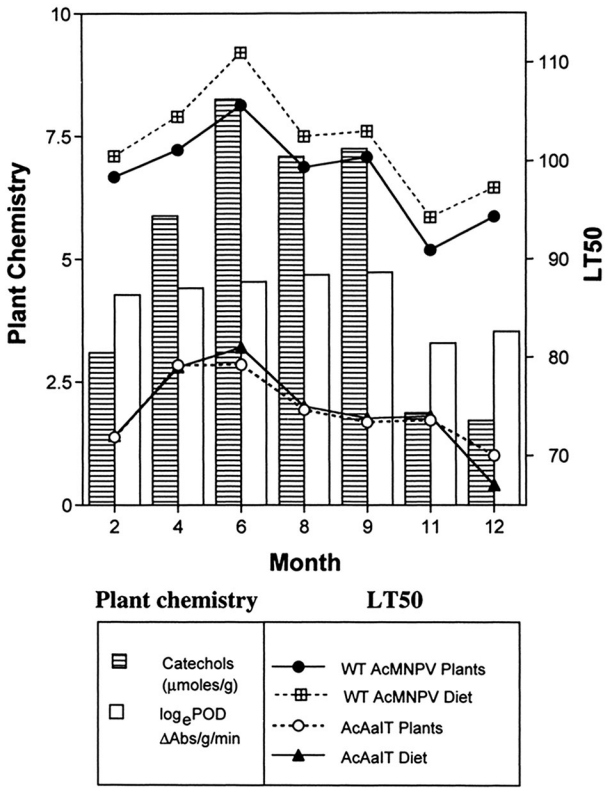

FIG. 3. Monthly variability in mean LT_{50} of *H. virescens* infected with wild-type AcMNPV or AcAaIT and fed on one of four host plants or artificial diet (controls) are shown as line graphs (right *Y* axis). Mean POD (solid bars) and catecholic phenolic levels (striped bars) of foliage are shown for each month (left *Y* axis). Month was a significant predictor of the survival curves for diet with WT AcMNPV (LRCS = 20.3, $P = 0.0024$), but the sign of the coefficients was opposite those for plants with one exception (Nov.). Despite the usefulness of month as a predictor of the survival curves of insects infected with AcAaIT and fed on plants (Table 7), there were no significant differences in monthly LT_{50}s for control insects (fed on diet) infected with AcAaIT (LRCS = 6.07, $P = 0.4160$).

dent variable on the survival times of neonate larvae of *H. virescens*, the dependent variable, infected with the WT AcMNPV or the recombinant baculovirus AcAaIT by fitting Cox's proportional hazards model. For both viruses, we first examined the relationship of each independent variable to the survival curve by entering each variable into the model separately. Then we fit the model using the best combination of variables. This procedure demonstrated some significant

TABLE 4. INFLUENCE OF EACH VARIABLE ON RATE OF MORTALITY OF *H. virescens* INFECTED WITH WILD-TYPE AcMNPV (IN ORDER OF RELATIVE IMPORTANCE) AND FED ON COTTON OR LETTUCE[a]

Variable	z statistic	Likelihood ratio (chi-square)	*df*	*P*
Month		70.2	6	<0.0001
Catechols	−4.78	24.6	1	<0.0001
POD	−4.75	24.0	1	<0.0001
Gossypol	−4.74	22.8	1	<0.0001
Plant type		19.2	3	0.0002[b]
Condensed tannins	−4.16	18.7	1	<0.0001
Protein	−3.99	16.2	1	<0.0001
Total phenolics	−3.70	14.9	1	0.0001
PPO	+3.20	10.0	1	0.0015

[a] Pooled survival data were analyzed for cotton and lettuce combined by Cox's proportional hazards model (N = 784).
[b] Insects fed on romaine lettuce died significantly faster than on the other plant types.

commonalities. For both viruses, rate of mortality varied with the levels of each phytochemical variable (Tables 4 and 5). For all chemical variables examined (except PPO), the higher the level, the slower the rate of mortality. In contrast, the higher the activity of PPO, the faster the insects died.

TABLE 5. INFLUENCE OF EACH VARIABLE ON RATE OF MORTALITY OF *H. virescens* INFECTED WITH AcAaIT (IN ORDER OF RELATIVE IMPORTANCE) AND FED ON COTTON OR LETTUCE[a]

Variable	z statistic	Likelihood ratio (chi-square)	*df*	*P*
Month		94.5	6	<0.0001
Condensed tannins	−4.68	23.6	1	<0.0001
Catechols	−4.32	20.0	1	<0.0001
Total phenolics	−4.00	17.6	1	<0.0001
PPO	+3.43	11.6	1	0.0006
Protein	−2.99	9.02	1	0.0027
POD	−2.92	8.81	1	0.0030
Gossypol	−2.72	7.46	1	0.0063
Plant type		7.02	3	0.0712

[a] Pooled survival data were analyzed for cotton and lettuce cultivars combined by Cox's proportional hazards model (N = 763).

When we examined the best combination of variables for predicting the survival curves, rate of mortality varied seasonally for both viruses (Tables 6 and 7). Although month was also a useful predictor of the survival curves for insects infected with WT AcMNPV that fed on diet, when the entire survival curve was modeled, the sign of the statistically significant coefficients for insects that fed on diet were opposite those for insects that fed on plants in the same months (with one exception, November; Table 6). Thus, month was not sufficient to explain the variability in the survival curves by itself. For the wild-type virus, in addition to month, rate of mortality varied with POD levels; plant type was not significant (Table 6). The higher the POD activity, the longer it took infected insects to die. Despite the fact that higher catecholic phenolic levels were strongly correlated with slower lethal times as a simple effect (Table 4), catechols dropped out of the final model (Table 6). This may be explained by the fact that catecholic phenolic content varied in parallel to month (Figure 3), and thus its presence in the model was probably redundant (i.e., multicollinearity).

For the recombinant virus AcAaIT, the best fit of the data required a more complex model (Table 7). POD, catecholic phenolics, condensed tannins, month, and plant type were predictive of rate of mortality. The higher the POD or phenolic level, the slower the insects died. Insects that fed on lettuce died

TABLE 6. BEST COMBINED MODEL FOR PREDICTING RATE OF MORTALITY OF *H. virescens* INFECTED WITH WILD-TYPE ACMNPV AMONG ALL PLANTS[a]

	Plants			Diet controls		
Variable	Parameter coeff.	z statistic	*P*	Parameter coeff.	z statistic	*P*
POD	−0.0007	−3.93	**<0.0001**			
Month (Feb. = 0)						
April	−0.2245	−3.25	**0.0012**	+2.27	+2.92	**0.0035**
June	−0.1202	−3.07	**0.0021**	+2.23	+2.74	**0.0062**
Aug.	+0.0116	+0.44	0.6600	+2.98	+3.74	**0.0002**
Sept.	+0.0235	+1.14	0.2600	1.35	+1.07	0.2900
Nov.	+0.1248	+6.05	**<0.0001**	1.99	+2.45	**0.0140**
Dec.	+0.0444	+2.99	**0.0028**	1.47	+1.24	0.2100

[a]Pooled survival data were analyzed for cotton and lettuce cultivars by Cox's proportional hazards model (Model: $\lambda = \exp(\beta X)$, where $\beta X = \exp[\beta_{month}(\text{Month}) - 0.0007(\text{POD})]$; LRCS = 87.1, $df = 7$, $P < 0.0001$, $N = 784$). *P* values for variables with coefficients that were significant at the 5% level are shown in bold. Despite a highly significant correlation between catecholic phenolics and survival times as a simple effect (chi-square = 24.6, $df = 1$, $P < 0.0001$; Table 4), this variable was not needed in the final model when month entered (catechols z = −1.01, $P = 0.3100$).

TABLE 7. BEST COMBINED MODEL FOR PREDICTING RATE OF MORTALITY OF *H. virescens* INFECTED WITH AcAaIT AMONG ALL PLANTS

Variable	Parameter coefficient	*z* statistic	*P*
POD	−0.0008	−1.99	**0.0470**
Catechols	−0.0413	−3.21	**0.0013**
Condensed tannins	−0.0066	−2.59	**0.0097**
Month (February = 0)			
April	−0.4379	−5.93	**<0.0001**
June	−0.0924	−2.16	**0.0031**
August	−0.0662	−2.02	**0.0440**
September	+0.1001	+4.48	**<0.0001**
November	−0.0063	−0.28	0.7800
December	+0.0504	+2.2	**0.0260**
Plant (Acala cotton = 0)			
Delta pine cotton	−0.0175	−0.27	0.7900
Romaine lettuce	+0.2197	+3.42	**0.0006**
Iceberg lettuce	+0.0868	+2.21	**0.0270**

[a] Pooled survival data were analyzed for cotton and lettuce cultivars combined by Cox's proportional hazards model $\{\lambda = \exp(\beta X)$, where $\beta X = \exp[\beta_{\text{month}}(\text{Month}) - 0.0008(\text{POD}) - 0.0413(\text{Catechols}) - 0.0065(\text{Tannins}) + \beta_{\text{plant type}}(\text{Plant type})]$; LCRS = 126, $df = 12$, $P < 0.0001$, $N = 763$]. *P* values for variables with coefficients that were significant at the 5% level are shown in bold. Romaine and iceberg lettuce required different intercepts compared with cotton, i.e., rate of mortality was significantly faster when insects were fed on lettuce than cotton.

significantly faster than insects that fed on cotton. Mortality was again slower in the summer months and faster in the fall and winter months (Table 7; Figure 3). Although month was a significant predictor of the survival curves for insects that fed on plants (Table 7), it was not significant for insects that fed on control diet despite the parallel relationship in LT_{50}s for insects that fed on diet or plants depicted in Figure 3. Because lettuce does not contain condensed tannins, it could be argued that inclusion of this variable in the model introduces bias. If tannins are removed from the model, month, plant type, POD ($\beta = -0.01$, $z = -2.47$, $P = 0.0140$), and catechol content ($\beta = -0.037$, $z = -2.90$, $P = 0.0037$) provided a useful, biologically meaningful model without specifying plant type (LRCS = 111, $df = 8$, $P < 0.0001$). Clearly, inclusion of POD level is very important because predicting larval mortality using a model containing only month and plant type had a lower likelihood ratio chi-square (LRCS) value than one that includes POD (LRCS for month + plant type + POD = 110, $df = 10$ vs. LRCS for month + plant type = 103, $df = 9$).

Separate Cotton and Lettuce Models. Because POD activities were always markedly higher in cotton than lettuce (Table 1), one might argue that bias is

introduced in the model because the data in essence represent two points of clustered data, which define a line. Thus, we analyzed cotton cultivars separately from lettuce and obtained models very similar to each other and to the combined model described above.

For insects infected with WT AcMNPV, the higher the POD and catecholic phenolic levels, the slower the insects died for both plant species (Table 8), also, the higher the gossypol content of cotton foliage, the slower the insects died. The full models for cotton and lettuce analyzed separately are similar to those for all plant types combined. On both cotton (Figure 4A) and lettuce (Figure 4B), rate of mortality varied with POD level and month (Table 9). For cotton, gossypol and catecholic phenolics were also needed in the model, although only gossypol had a statistically significant z statistic at the 5% level (Table 9). POD was not significant in the absence of catecholic phenolics. Thus, in agreement with the model using all plant types combined (Table 6), the higher the POD activity of each plant species, the slower the insects died (Figures 4A and 4B).

Relationships between individual variables and rate of mortality of insects infected with AcAaIT that fed on cotton (Table 10) were also similar to those obtained using data for all plants combined. Again the higher the POD, catecholic phenolics, gossypol, and condensed tannin levels of cotton foliage, the slower the insects died (Table 10). For lettuce, however, only POD was significantly correlated (negative coefficient) with rate of mortality by itself (Table 10). The most robust full models required inclusion of month for both plant species (Table 11). However, the inclusion of other variables in addition to month was not required to explain the variability in survival times for insects

TABLE 8. INFLUENCE OF EACH VARIABLE ON RATE OF MORTALITY OF *H. virescens* INFECTED WITH WILD-TYPE AcMNPV AND FED ON COTTON OR LETTUCE[a]

	Cotton		Lettuce	
Variable	z statistic	P	z statistic	P
Month		**<0.0001**		<0.0001
Catechols	−2.86	**0.0043**	−1.46	**0.0150**
POD	−3.19	**0.0140**	−5.85	**<0.0001**
Total phenolics	−1.55	0.1200	−0.43	0.0670
Gossypol	−2.93	**0.0034**		NA
Condensed tannins	−1.91	0.0570		NA
PPO		NA	+0.26	0.7960

[a]Survival data for the two cultivars each of cotton and lettuce were analyzed separately by Cox's proportional hazards model. P values for variables with coefficient that were significant at the 5% level are shown in bold. NA = not applicable.

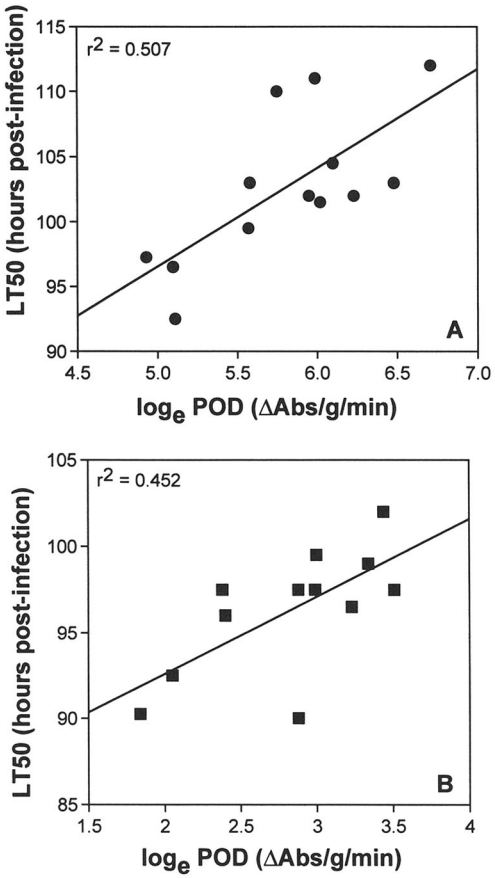

FIG. 4. Influence of peroxidase (POD) level on the LT_{50} of insects infected with WT AcMPV and fed on cotton (A) or lettuce (B) foliage. LT_{50}s were determined by the Kaplan-Meier estimator.

fed on cotton (Table 11). For lettuce, there were two equally useful models. In separate models PPO and POD were each negatively correlated with rate of mortality (Table 11). If PPO was in the model, it was also necessary to include noncatecholic phenolics (negative coefficient) and catecholic phenolics (positive coefficient) to describe the survival curves, although their z statistics were not significant in the final model. Similarly, if POD was in the model, it was also necessary to include catecholic phenolics (negative coefficient) to help explain some of the variability despite the fact that the z statistic for catechols was not significant at the 5% level in the final model.

TABLE 9. BEST MODELS FOR PREDICTING RATE OF MORTALITY OF *H. virescens* INFECTED WITH WILD-TYPE AcMNPV FED ON COTTON OR LETTUCE[a]

	Cotton			Lettuce		
Variable	Parameter coeff.	*z* statistic	*P*	Parameter coeff.	*z* statistic	*P*
Month			**<0.0001**			**<0.0001**
POD	−0.3420	−3.19	**0.0014**	−0.259	−1.98	**0.0470**
Catechols	−0.0255	−1.05	0.3000			
Gossypol	−0.2223	−2.06	**0.0400**			

[a]Survival data for cotton and lettuce were analyzed separately by Cox's proportional hazards model [$\lambda = \exp(\beta X)$]. Cotton model: $\beta X = \exp[\beta_{month}(\text{Month}) - 0.342(\log_e \text{POD}) - 0.0255(\text{Catechols}) - 0.2223(\text{Gossypol})]$; LRCS = 32.8, $df = 9$, $P = 0.0001$, $N = 380$; Lettuce model: $\beta X = \exp[\beta_{month}(\text{Month}) - 0.259(\log_e \text{POD})]$; LRCS = 53.5, $df = 7$, $P < 0.0001$, $N = 404$, *P* values for variables with coefficients that were significant at the 5% level are shown in bold.

TABLE 10. INFLUENCE OF EACH VARIABLE ON RATE OF MORTALITY OF *H. virescens* INFECTED WITH AcAaIT AND FED ON COTTON OR LETTUCE[a]

	Cotton		Lettuce	
Variable	*z* statistic	*P*	*z* statistic	*P*
Month		**<0.0001**		**<0.0001**
Catechols	−3.89	**<0.0001**	−0.71	0.4800
POD	−3.56	**0.0004**	−3.48	**0.0005**
Total phenolics	−3.27	**0.0011**	−0.58	0.5600
Gossypol	−1.99	**0.0470**		NA
Condensed tannins	−4.63	**<0.0001**		NA
PPO		NA	+0.86	0.3920

[a]Survival data for cotton and lettuce were analyzed separately by Cox's proportional hazards model. *P* values with coefficients that were significant at the 5% level are shown in bold. NA = not applicable.

DISCUSSION

Plant phenolase activity influenced the progression and severity of baculoviral disease in noctuid larvae in a nonlinear, highly context-dependent fashion. Furthermore, in the plants examined, POD had a greater and more consistent role than PPO in inhibiting baculoviral disease (to aid the reader models are

TABLE 11. BEST MODELS FOR PREDICTING RATE OF MORTALITY OF *H. virescens* INFECTED WITH AcAAIT AND FED ON COTTON OR LETTUCE[a]

	Cotton			Lettuce		
Variable	Parameter coeff.	z statistic	P	Parameter coeff.	z statistic	P
Month			**<0.0001**			**<0.0001**
PPO				−0.0597	−2.05	**0.0410**
Catechols (model 1)				+0.0875	+1.30	0.1900
Catechols (model 2)				−0.0495	−1.46	0.1400
Noncatechols[b]				−0.0374	−1.58	0.1100
POD				−0.0423	−2.14	**0.0320**

[a]Survival data for cotton and lettuce were analyzed separately by Cox's proportional hazards model. P values for variables with coefficients that are significant at the 5% level are shown in bold. For cotton, only month was significant (LRCS = 78.5, $df = 6$, $P < 0.0001$, $N = 363$). For lettuce there were two equally useful models: $\lambda = \exp(\beta X)$ where βX for model 1 = $\exp[\beta_{month}(\text{Month}) - 0.0597(\text{PPO}) + 0.0875(\text{Catechols}) - 0.0374(\text{Noncatechols})]$; LRCS = 60.3, $df = 9$, $P < 0.0001$, $N = 400$. βX for model 2 = $\exp[\beta_{month}(\text{Month}) - 0.0423(\log_e \text{POD}) - 0.0495(\text{Catechols})]$; LRCS = 58.6, $df = 8$, $P < 0.0001$, $N = 400$.

[b]Noncatechols = total phenolics − catecholic phenolics.

presented as schematics in Figures 5 and 6). In addition to the importance of plant phenolase activity as a modulator of baculoviral disease, several important concepts were reinforced concerning the means by which host plants influence disease—because the influence of phytochemicals on viral disease depends on chemical context, the impacts of phytochemicals are multiplex, interactive, and multifunctional.

Multiplicity. The influence of host plant on viral efficacy (measured as the probability of dying or time to death) is multivariate. Predicting the impact of phytochemicals on disease required regression of multiple independent variables, not single variables. For example, larval mortality on lettuce could be predicted from PPO, catecholic phenolics, or noncatecholic phenolic levels (and viral dose). (Figure 5). However, analysis of the influence of these individual phytochemicals on baculoviral disease explained only a part of the story. When these phytochemical variables were analyzed together (as a full model), we obtained a better fit of the data (chi-square for individual variables 16.5, 12.8, 5.5, and 248, respectively vs. chi-square = 273 for full model; Table 3, Figure 5). In addition, consideration of the impact of multiple phytochemicals on viral disease allowed us to construct rational hypothetical mechanisms for the inhibition of viral disease, the veracity of which can be subjected to further investigation (see discussion below on biological interpretation of the models).

Interactivity. A second major concept to be gleaned from this study is that

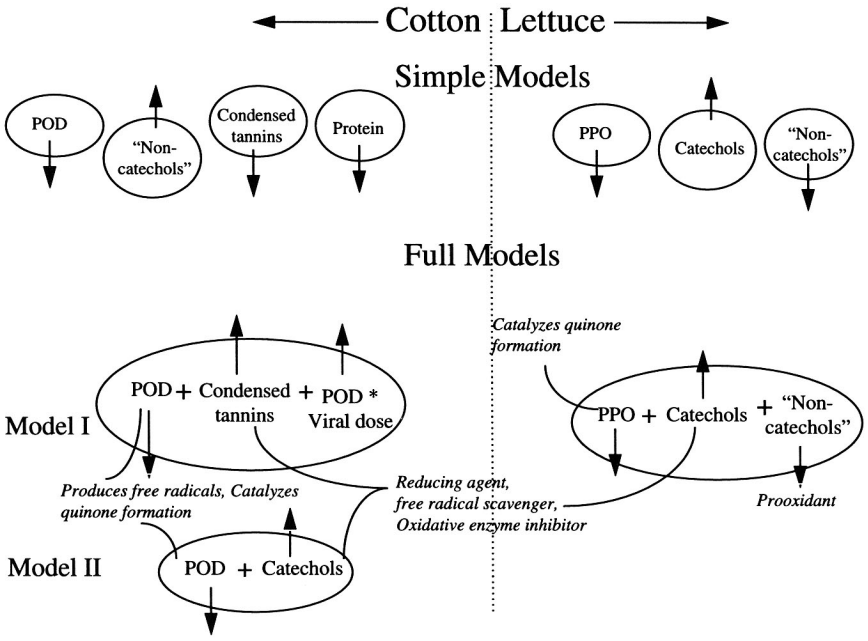

FIG. 5. Schematic representation of the impact of phytochemicals on lethal infection caused by the wild-type baculovirus AcMNPV in *H. virescens*. Arrows pointing upward or downward increase or decrease, respectively, the probability of an insect dying from viral infection. Note that some variables have different effects whether they are considered separately (simple regression, separate circles) or in combination with other phytochemicals (full models, multiple variables within same circle).

the impact of a given phytochemical on viral disease depended upon its interaction with other phytochemicals. Most illustrative of this concept was the influence of different classes of phenolics on viral disease. There were occasions where the impact of a given phytochemical variable changed (sign of the β coefficient) when it was considered in isolation versus in combination with other phytochemicals, i.e., in context. For example, condensed tannins were correlated with decreased larval mortality as a simple effect. In contrast, tannins were correlated with increased larval mortality in the context of POD activity.

As a simple effect, our finding that inhibition of lethal infection by increased tannin content of cotton foliage is in agreement with other studies that have shown a relationship between hydrolyzable and/or condensed tannins and a decrease in lethal infections caused by the *Lymantria dispar* NPV (LdMNPV) or *Helicoverpa zea* NPV (HzSNPV) (Keating et al., 1988, 1989; Schultz and Keating, 1991; Young et al., 1995). However, these studies either incorporated

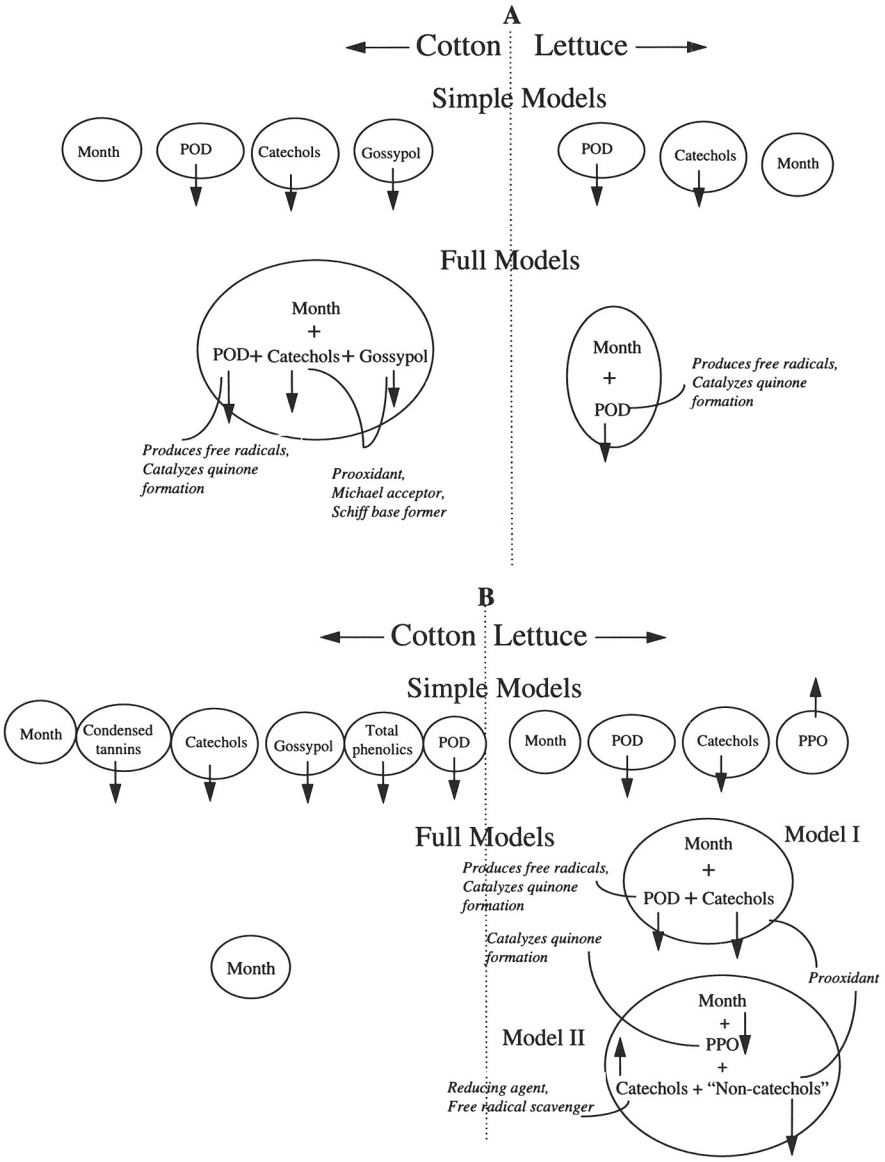

FIG. 6. Schematic representation of the impact of phytochemicals on rate of mortality of neonate larvae of *H. virescens* infected with either (A) the WT AcMNPV or (B) the recombinant AcAaIT. Arrows pointing upward or downward increase or decrease, respectively, the speed of kill. Note that some variables have different effects whether they are considered separately (simple regression, separate circles) or in combination with other phytochemicals (full models, multiple variables within same circle).

different concentrations of tannin into artificial diet or considered the impact of tannins in foliage in the absence of other phytochemicals, such as their possible interaction with plant oxidative enzymes and/or other types of phenolic compounds in foliage. Given that condensed tannins in our study were correlated with an opposite effect on viral disease when considered in isolation versus in the context of admixture, we suggest that estimating the impact of a phytochemical on viral disease out of context may lead to misleading conclusions.

A second example of interactions influencing viral disease was the impact of the host plant in the context of viral dose. Insects treated with a baculovirus were more likely to die from viral infection when fed on cotton than when fed on lettuce, with the greatest impact of the host plant on lethal infection at lower viral doses. Furthermore, POD interacted with viral dose such that the higher the viral dose, the less influence POD had in decreasing larval mortality on cotton. Differential impacts of phytochemicals on larval susceptibility to viral infection at low but not high viral doses was also reported by Felton et al. (1987). Furthermore, the absence of significant host plant effects on percent mortality at the highest viral dose (LD_{99}) suggests that when viral inoculum builds to levels capable of initiating an epizootic in natural environments, host plant chemistry probably has little if any effect on whether the insect will ultimately die from viral infection but may still influence the rate of mortality. The viral dose for these experiments represented the LD_{99}.

Multifunctionality. A final concept that can be inferred from our results is that the same phytochemical class can function by more than one mechanism in its impact on viral activity; the particular mechanism assumed is dependent upon context (admixture). This concept is well exemplified by the impact of catecholic phenolics on percent or rate of mortality. Catecholic phenolics increased the probablity of an insect dying from viral infection in both cotton and lettuce. In contrast, catecholic phenolics were correlated with slower lethal times, with one exception—insects infected with the recombinant virus AcAaIT died faster the higher the catecholic phenolic content of lettuce. In another instance, noncatecholic phenolics appeared to enhance viral disease (correlated with increased mortality) on cotton, whereas this variable appeared to attenuate disease on lettuce. Thus, a given phytochemical may enhance or attenuate viral disease depending upon context.

Biological Interpretation of the Models. How plant phenolase activity inhibits the ability of baculoviruses to produce lethal infections in larval hosts is not known, but we now have sufficient information to begin exploration of some potential mechanisms. In the plants examined, POD had a greater and more consistent role than PPO in exerting a negative influence on viral efficacy. Since both enzymes convert a variety of phenolics to highly reactive quinone end products, why is POD more influential?

There are several possible explanations for the more influential role of POD

in diminishing viral disease. Firstly, POD oxidizes a much broader range of substrates than does PPO, including intact proteins (Singleton, 1987; Robinson, 1991), thereby increasing the diversity of reaction products that may negatively affect the baculovirus. Secondly, the midgut of heliothines is nearly anaerobic (K. S. Johnson, personal communication), which may limit the available O_2 for PPO-mediated reactions. Reactions catalyzed by PPO are O_2 dependent, whereas many reactions (including phenolic oxidation) catalyzed by POD are O_2 independent (but H_2O_2 dependent) (Singleton, 1987; Robinson, 1991). Thirdly, POD may be more stable in the midgut of noctuid larvae than PPO; thus, POD activity may be further enhanced by the production of H_2O_2 by salivary glucose oxidase in oral secretions of heliothines (G. W. Felton, personal communication). In addition to the above, we believe that a critical process leading to the attenuation of viral disease by POD is the generation of free radicals.

POD oxidizes phenolics to quinones by a one-electron transfer mechanism employing H_2O_2 as a cosubstrate. The mechanism of oxidation produces a highly reactive semiquinone free radical intermediate, which can then initiate further propagation of free radicals (Butt, 1981; Butt and Lamb, 1981; Ahmad, 1995; Bi et al., 1997). We propose this intermediate is the most significant negative factor leading to inhibition of baculoviral disease. In contrast, PPO directly produces the quinone using molecular oxygen as a cosubstrate (Mayer, 1987), which, if our hypothesis is correct, assumes that the quinone is much less detrimental to the virus because it is less effective at secondarily producing free radicals by redox cycling. It is also possible that redox cycling initiated by POD generates active oxygen species (AOS) that are detrimental to the virus.

Because the polyhedral occlusion body (OB) of the baculovirus is primarily composed of protein (Vlak and Rohrmann, 1985; Whitt and Manning, 1987, 1988) with oxidizable and alkylatable amino acid residues (e.g., tyrosine, lysine, cysteine, methionine, and histidine), the OB is subject to covalent binding by reactive end products such as quinones formed by phenolic oxidation catalyzed by phenolases (Pierpoint, 1983; Felton et al., 1992). Although it has been shown that covalent binding can occur between oxidized phenolics, such as chlorogenoquinone formed by PPO activity and the OB in vitro (Felton and Duffey, 1990), it is not known whether this process has any effect on the ability of baculoviruses to infect their hosts on intact foliage.

Viral disease may also be attenuated via processes initiated by POD in foliage, such as oxidation of phenolics and other reaction products that produce free radicals that attack proteins and lipids (Summers and Felton, 1994). These processes may generate oxidative stress in the insect midgut leading to damage of the infected gut cells. Damaged midgut cells containing virus may be sloughed before the virus can spread beyond the midgut, effectively eliminating the infection from the insect.

Interactions between plant phenolases and phenolic substrates support the

notion that plant constituents influence viral disease in a manner that is multiplex, interactive, and multifunctional, each depending upon context. For example, the influence of different classes of phenolics was highly dependent upon context. Thus, the distinction between catecholic and noncatecholic phenolics (i.e., monohydroxyphenolics) is both chemically and biologically relevant in our experimental system. The two enzymes, PPO and POD, show a marked preference for oxidizing catecholic phenolics (Felton et al., 1989). For example, PPO readily oxidizes *o*-dihydroxyphenolics, such as chlorogenic acid, caffeic acid, and (±)-catechin, but has no activity on the monohydroxyphenolics, ferulic, *p*-coumaric, or *p*-hydroxybenzoic acids (Butt, 1981; Singleton, 1987). In addition, we observed 13-fold and 7-fold increases in the PPO and POD activities, respectively, of several plants during oxidation of catecholic phenolics in mixture, including chlorogenic acid plus rutin or catechin, compared to during oxidation of each phenolic species in isolation (Hoover et al., unpublished). In contrast, PODs from several plants have potent abilities to oxidize monohydroxyphenolics (Robinson, 1991), although PODs from cotton and tomato were more active on *o*-dihydroxyphenolics both singly and in mixture than on monohydroxyphenolics (Hoover et al., unpublished). Hence, the ratios and absolute amounts of catecholic phenolics and monohydroxyphenolics are important elements determining oxidative damage to viruses.

In general, phenolics, particularly some of the catecholic phenolics, have a variety of reactivities that, in a basic environment and/or in the presence of PPO or POD, include antioxidant activities such as radical and electrophile scavenging and reducing power [e.g., chlorogenic, caffeic, and ferulic acids (Huang and Ferraro, 1992) and tannins (Huang et al., 1992)], prooxidative activity (initiation of chain reactions generating AOS, organic free radicals, and/or quinones, e.g., quercetin), and chelation (Ho et al., 1992; Huang et al., 1992; Ahmad, 1995). The degree and proportion to which they exhibit these reactivities appear to be dependent upon context, identity, and dose. For example, under aerobic conditions and in the presence of trace metals, quercetin can produce superoxide and other AOS (Ho et al., 1992; Huang et al., 1992; Ahmad, 1995). However, in another context quercetin and other flavanoids such as rutin can also scavenge superoxide anions (Robak and Gryglewski, 1988). The reactivity of phenolic species may also be dose-dependent in a manner similar to that of ascorbate. Ascorbate can function as a prooxidant or antioxidant, depending on its concentration (Englard and Seifter, 1986; Frei et al., 1989; Felton, 1995; Felton and Summers, 1995). Thus, it is possible that catecholic phenolics in lettuce and condensed tannins in cotton are serving as reducing agents and/or free radical scavengers, thus protecting the virus from inactivation by oxidative activity. Furthermore, because tannins and other phenolics are known to act as competitive inhibitors of PPO and POD (Golan-Goldhirsh and Whitaker, 1984; McEvily et al., 1992), it is possible that these phytochemicals can also

enhance viral disease in combination with oxidative enzymes by inhibiting oxidative enzyme activity, thereby protecting the virus from inactivation. In contrast, noncatecholic phenolics were correlated with decreased mortality on lettuce, suggesting that a variety of such phenolic compounds in lettuce may serve as substrates for oxidative enzymes with subsequent negative impact on viral activity.

Despite the problem of multicollinearity in some models, we believe inclusion of phenolics in these models is necessary to obtain a more complete understanding of the impact of host plant on viral efficacy. Not only is multicollinearity common in regression models (Neter et al., 1990), but it was expected, given the conditions of our study involving growing plants at different times of year under natural conditions (Karban, 1987; Bryant et al., 1988; Chaves et al., 1997). Furthermore, we could not eliminate multicollinearity by excluding month from some models because it was sometimes required to explain the variability in the remaining phytochemicals to discern the importance of phenolics in influencing viral disease. More importantly, inclusion of phenolics in our models makes sense biologically. It is possible that increasing amounts of catecholic phenolics beyond a minimum level may not be necessary to inactivate (or enhance) viral efficacy. This is because enzymes are only required in catalytic amounts and there is an excess of phenolic substrates in these plants. It is also possible that increasing catecholic phenolic levels beyond a minimum concentration is inconsequential for the virus. Moreover, high phenolic levels can even interfere with enzyme activity, a phenomenon often referred to as autoinhibition (Golan-Goldhirsh and Whitaker, 1984). Assuming redox reactions that generate free radicals are involved as a potential mechanism of viral inactivation in these systems, and because these reactions are propagative, small amounts of phenolics may be all that is required in the presence of high enzymatic activity for viral inactivation to occur.

The mechanism(s) whereby plant phenolase activity influences speed of kill after infection by baculoviruses is also unknown. We suspect that under the conditions of this study, we may be witnessing a postinfectional impact on speed of kill of the larval host mediated through the physiological status of the insect in response to interactions between the insect and phytochemicals, not a direct effect of phytochemicals on the virus. Phenolase activity may retard the rate of mortality by affecting the growth rates of infected insects. In a previous study, we found that slower growth rates of *H. virescens* infected with AcMNPV were strongly correlated with slower speeds of kill (Hoover et al., 1996). Thus, it is possible that oxidative enzymes and phenolic compounds normally correlated with antinutritive effects in noctuid larvae (Felton et al., 1992; Duffey and Stout, 1996) have the inadvertent effect of slowing lethal times of infected insects.

In addition to host plant effects, seasonal differences in physiological susceptibility to viral infection likely contributed to the monthly differences in

survival times of insects treated with baculovirus. Month was a significant predictor of the survival curves of insects infected with WT AcMNPV and fed on artificial diet, although month was not a significant predictor of the survival curves for AcAaIT on diet. A variety of biotic and abiotic factors have been shown to influence susceptibility of insects to entomopathogens including differences in genetic susceptibility to viruses among conspecific strains of insects (Watanabe, 1987).

Research is currently underway in our laboratory to determine the mechanisms of the differential ability of POD and PPO to inhibit baculoviral disease, to determine the interaction of these enzymes with various classes of phenolic compounds in this process, and to distinguish free radical from enzymatic effects on viral activity. By manipulating phytochemicals in admixture we hope to establish a better understanding of the mechanism(s) whereby phytochemicals influence viral efficacy among different host plants. The antiviral effects of these phytochemicals may have profound consequences for the compatibility of baculoviruses with host plants expressing these plant defensive compounds at high levels. Knowing the mechanism(s) whereby phytochemicals inhibit baculoviral disease will facilitate a more rational exploration of formulation chemistry that may mitigate these impacts.

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INFLUENCE OF INDUCED PLANT DEFENSES IN COTTON AND TOMATO ON THE EFFICACY OF BACULOVIRUSES ON NOCTUID LARVAE

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Abstract—Constitutive phenolase activity of plants has a profound ability to modulate disease in insects caused by baculoviruses. We investigated the influence of damage-induced plant phenolic oxidases in cotton and tomato on mortality caused by two different baculoviruses in their respective hosts, *Heliothis virescens* (L.) and *Helicoverpa zea* (Boddie). For both plant species, peroxidase (POD) and phenolic levels were predictive of larval mortality caused by baculoviruses. The higher the POD activity, the lower the mortality in both hosts. Different classes of phenolics (e.g., monohydroxyphenolics vs. catecholic phenolics) in combination with POD activity had different effects on the severity of viral disease depending upon mixture, which implies that viral efficacy is predictable only if total chemical content of the plants is specified. Inhibition of baculoviral disease by plant phenolase activity has potential implications for the compatibility of baculoviruses with induced resistance in IPM programs.

Key Words—Induced resistance, baculovirus, *Heliothis virescens*, *Helicoverpa zea*, peroxidase, polyphenol oxidase, oxidative enzymes, tritrophic interactions.

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INTRODUCTION

For the past few decades, the field of plant–insect interactions has been the subject of vigorous discussion of the utility of induced plant natural products as putative defenses against herbivorous insects (Karban and English-Lobe, 1988; Duffey and Felton, 1989; Felton et al., 1989; Karban and Myers, 1989; Tallamy and Raupp, 1991; Wolfson, 1991; Baldwin, 1994). When plants are damaged by insect feeding, the plant responds with *de novo* synthesis and activation of a suite of phytochemicals, some of which may defend the plant against herbivores (Duffey and Felton, 1989; Baldwin, 1994; Duffey and Stout, 1996). Many of these induced changes involve activation and/or alteration of enzymatic activity (Duffey and Felton, 1989, 1991; Felton et al., 1989, 1994; Felton and Summers, 1993; Duffey and Stout, 1996). Although such changes are often a first line of defense against invasive organisms, their impact on these organisms is often difficult to predict. It is particularly difficult to predict the ecological consequences of induction when one includes the impact of induced plant responses on third trophic level organisms (Hunter and Schultz, 1993).

There is ample evidence that the constitutive and induced chemical composition of plants can have a major impact on the ability of predators and parasitoids to utilize their insect hosts (Campbell and Duffey, 1981; Duffey and Bloem, 1986; Duffey et al., 1986; Price, 1986; Barbosa, 1988; Bloem et al., 1989; Bozer et al., 1996; Stamp et al., 1997; Traugott and Stamp, 1997). In addition, many phytochemicals that mediate plant–insect interactions have marked antagonistic effects on microorganisms (McClure, 1975; Mucsi et al., 1977; Jones, 1984; Berenbaum, 1988). There is minimal information available, however, on the influence of induced plant responses on entomopathogens. Hunter and Schultz (1993) demonstrated that gypsy moth larvae were less susceptible to the *Lymantria dispar* NPV consumed on foliage from branches that had been subjected to increasing levels of defoliation. Despite the fact that prior herbivory induced higher levels of gallotannins (which generally benefits the plant), in this case induced foliage may offer an advantage to the herbivore by somehow making it less susceptible to the virus. Consequently, it is not possible to glean a comprehensive understanding of ecological theory without inclusion of the impact of induced plant responses on third trophic level organisms (Price et al., 1980). Furthermore, examination of the influence of induced plant responses on entomopathogens is critical for assessing the compatibility of using induced resistance and entomopathogens as simultaneous tactics in an IPM program.

Plant oxidative enzymes are suspected, but not proven, to play a significant role in inhibiting disease by baculoviruses (Felton and Duffey, 1990; Duffey et al., 1995; Hoover et al., 1998). For example, we recently found that plant

constitutive phenolase activity is, at least in part, responsible for inhibiting percent and rate of mortality of larval *H. virescens* treated with a baculovirus and that the baculovirus of the alfalfa looper, *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) was two to three times more infective on iceberg or romaine lettuce than on two different cotton cultivars (Hoover et al., 1998). The impact of these host plants on mortality of larval *H. virescens* treated with AcMNPV could be predicted from the level of phenolics and the activity of the oxidative enzymes peroxidase (POD) or polyphenol oxidase (PPO). In the present study, we used the inherent differences in type and degree of inducibility of PPO and POD between cotton and tomato to test the hypothesis that plant phenolase activity can inhibit baculoviral disease. Using two different types of damage (insect feeding and UV irradiation), we found that larval mortality was significantly lower when virus was consumed on foliage in which POD was induced. Induction of PPO by itself had no effect on larval mortality.

METHODS AND MATERIALS

To evaluate the influence of plant phenolase activities and phenolic substrates on the efficacy of baculoviruses against noctuid larvae, we induced plants to express higher PPO activity in tomato and POD activity in tomato and cotton foliage. We then examined the impact of these differential inductions on baculoviral disease.

Plants. Cotton (*Gossypium hirsutum* cv. Acala SJ2) and tomato (*Lycopersicum esculentum* cv. Bonnie Best) were grown in a greenhouse under natural conditions. The photoperiod was 16L:8D and the temperature range was 24–30°C. We did not use artificial lighting. Induction of POD in cotton was accomplished by confining a fourth-instar *H. virescens* to the third leaf of four-leaf-stage plants for 16 hr. Induction of PPO in tomato was achieved by confining a fourth-instar *Helicoverpa zea* to the terminal leaflet of the third leaf of four-leaf stage plants for 8 hr. POD was induced in tomato by exposing four-leaf stage plants to UV irradiation for 4.5 hr from a pair of 40-W fluorescent lamps (Phillips TL40W/12) suspended approximately 25 cm above the plants. The spectral characteristics of these lamps are specified in Caldwell et al. (1986). Assays were performed 48 (tomato) or 96 hr (cotton) after initiation of damage. Chewing damage leads to maximum induction of PPO in tomato (Stout et al., 1994) and POD in cotton (Bi et al., 1997) within 24–48 and 72–96 hr, respectively. For all bioassays there were three experimental groups: induced treatment, uninduced treatment (plant control), and a diet treatment (diet control). Chemical and biological assays were conducted with the third leaf of the cotton

plants or the leaflets adjacent to the terminal leaflet of the third leaf of the tomato plants. Insects in the diet control group received their viral dose on an 8-mm^3 cube of artificial diet (BioServ, Inc.).

Insects. Eggs of *H. virescens* and *H. zea* were obtained from the United States Department of Agriculture Agricultural Research Station (Stoneville, Mississippi). Neonate larvae were reared to third instar individually on 8 cm^3 of semisynthetic diet (BioServ, Inc.) in 24-well tissue culture plates (Fisher, St. Louis, Missouri) at 26 ± 1°C and 16L:8D.

Viruses. The baculoviruses AcMNPV (C6 clone) (Ayers et al., 1994) and *H. zea* single nucleocapsid NPV (HzSNPV, original isolate plaque purified from Elcar, Sandoz-Wander, Wasco, California) were amplified in larvae of *H. virescens* and *H. zea*, respectively, and extracted, partially purified, and stored until use as described in Hoover et al. (1995).

Foliar Chemical Assays. Plants were assayed for chemical content on the same day that foliage was used for bioassay. PPO and POD activities were determined colorimetrically with caffeic acid and guaiacol/H_2O_2, respectively, as substrates. Activities were measured as the increase in OD_{470} per minute per gram (Ryan et al., 1982; Felton et al., 1989). We attempted to measure PPO activity in cotton with a diversity of substrates, including chlorogenic acid, caffeic acid, catechol, (±)-catechin, quercetin, rutin, and 2,3-dihydroxybenzoic acid. Catecholic phenolic content was determined colorimetrically at OD_{390} with a 0.5% diphenylborinic acid–ethanolamine complex and chlorogenic acid as the standard (Broadway et al., 1986; Felton and Duffey, 1990). Total phenolics were measured with the Folin-Ciocalteau reagent and chlorogenic acid as a standard (Singleton and Rossi, 1965). We calculated noncatecholic phenolics as the difference between total phenolics and catecholic phenolics.

Bioassays. We used two different insect–virus–plant systems to examine the impact of plant oxidative enzymes and phenolic substrates on mortality of noctuid larvae caused by baculovirus. Larvae of *H. virescens* were treated with the baculovirus AcMNPV on cotton, and *H. zea* larvae were dosed with the baculovirus HzSNPV on tomato. We were not able to test reciprocal relationships because this strain of *H. virescens* will not eat tomato foliage and *H. zea* is only marginally permissive to infection by AcMNPV.

Within 6 hr after molting to the third instar, larvae were removed from diet and starved overnight to void gut contents. Leaf disks of 0.5 cm diameter were cut from foliage of each plant and placed on agar in 24-well tissue culture plates. There were five to six tomato plants and six to eight cotton plants in each treatment group used for each replicate. There were two treatment factors: (1) **chemical state**, for delivery of viral inoculum that utilized induced plants, untreated control plants, and artificial diet controls; and (2) **viral dose**, delivered as three to four doses depending upon the experiment. Induced or uninduced

leaf disks from each treatment group were distributed equally among all viral doses for each plant treatment.

Polyhedral occlusion bodies (OBS) were suspended in double-distilled H_2O at different concentrations for application in 1-μl aliquots to each leaf disk. Insects received one of four doses representing the approximate LD_{99}, LD_{75}, LD_{50}, or LD_{25} determined via artificial diet. For AcMNPV in *H. virescens* the doses were 3000, 300, 100, or 30 OBS/larva and for HzSNPV in *H. zea* the doses were 400, 20 (insect chewing damage experiment only), 7, or 3 OBS/larva. A larva was transferred individually to a leaf disk after allowing viral inoculum to dry at ambient temperature. After 18 hr, insects that consumed an entire treated leaf disk or treated artificial diet were transferred individually to excess artificial diet in 35-ml cups and maintained until death or pupation at 26 $\pm$ 1°C and 16L:8D. Mortality was scored at eight to nine days after infection. There were 35–45 larvae per dose per treatment and the experiment was replicated five to six times.

Data Analysis. Data were analyzed by logistic regression to determine if the probability of an insect dying could be predicted from plant chemical levels (Kalbfleisch and Prentice, 1980; Collett, 1994). Each variable was regressed separately followed by backward stepwise logistic regression to determine the most useful model. The model estimates the unknown parameter coefficients as follows: $\log(p/1 - p) = \beta X$, where $\beta X = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \ldots$ Therefore, the probability of dying $(p) = \exp\{(\beta X)/[1 + \exp(\beta X)]\}$. Thus, parameter coefficients (β) with a positive sign indicate a variable that increases the probability of an insect dying; negative coefficients decrease the probability of dying.

Mean oxidative enzyme and phenolic levels of induced and uninduced foliage used for bioassays were compared within each replicate by Student's *t* test (Steel and Torrie, 1980). Factorial ANOVA was used to compare percent mortality at each dose among treatments. Percentages were subjected to arcsin transformation and means separated by Fisher's PLSD.

RESULTS

Influence of Induction of POD in Cotton on Mortality of H. virescens *by AcMNPV.* Chewing-damage by larvae of *H. virescens* significantly induced POD activity in damaged cotton leaves an average of fourfold compared with undamaged controls (Figure 1). As a consequence, mortality of larval *H. virescens* dosed with AcMNPV was significantly lower on POD-induced cotton foliage compared to mortality on uninduced foliage at all viral doses by an average of 37% (Table 1). In addition, there was a differential effect of induced POD according to viral dose (Table 1). The lower the viral dose, the greater the

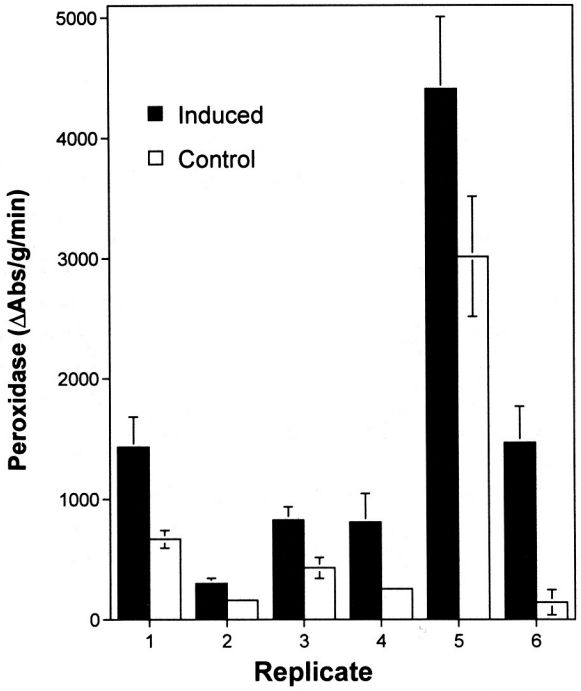

FIG. 1. Induction of peroxidase (POD) activity in cotton foliage by chewing damage by *H. virescens*. There were six replicates, done at different times. Bars represent the mean POD activity of six to eight plants per treatment per replicate. Error bars are the standard error of the mean (some errors too small to portray). POD activity was significantly higher in damaged foliage within each replicate at the 5% level.

TABLE 1. EFFECT OF INDUCTION OF POD IN COTTON ON MORTALITY OF *H. virescens* LARVAE DOSED WITH AcMNPV AT 1 OF 4 VIRAL DOSES[a]

	Mortality (%)			
	30 OBS/larva	100 OBS/larva	300 OBS/larva	3000 OBS/larva
Induced cotton	12.2a	16.3a	30.4a	67.7a
Uninduced cotton	26.8b	27.6b	46.4b	81.9b
Diet	31.5c	48.6c	70.9c	94.3c

[a]OBS = occlusion bodies. Mean percentage mortality of six replicates with 35–45 larvae of *H. virescens* per replicate. Means within columns followed by different letters are significantly different at the 5% level according to Fisher's PLSD. ANOVA: treatment $F = 11.90$, $df = 2, 57$, $P < 0.0001$; dose $F = 34.7$, $df = 3, 57$, $P < 0.0001$.

reduction in mortality on damaged foliage. At LD_{25} and LD_{50}, mortality was reduced by 55 and 41%, respectively. At LD_{75} and LD_{99}, mortality was reduced by 35% and 17%, respectively. Mortality also was significantly lower on cotton in any state than on artificial diet. PPO activity was not detectable in cotton foliage.

The higher the POD (Figure 2) or catecholic phenolic levels of induced or uninduced cotton foliage (Table 2), the lower the probability of an insect dying from viral infection. In addition, the higher the noncatecholic phenolic level, the higher the probability of dying from viral infection (Table 2).

Chewing damage did not significantly alter plant phenolic levels in four of six replicates. However, in two replicates, induced foliage had 1.5–2× higher levels of total phenolics (43 vs. 21 μmol/g and 15 vs. 9.9 μmol/g, respectively, $t = 2.9$, $P = 0.0096$ and $t = 2.7$, $P = 0.0200$, respectively). Catecholic phenolics were 2× higher in uninduced foliage in one replicate (9.4 vs. 4.8 μmol/g, $t = 3.9$, $P = 0.0012$). Mean catecholic and total phenolic levels (mean ± SE) of induced vs. uninduced foliage were 5.5 ± 0.7 vs. 5.9 ± 1.4 and 16 ± 2.1 vs. 20 ± 4.1 μmol/g, respectively (N = 6–8 plants/treatment/replicate).

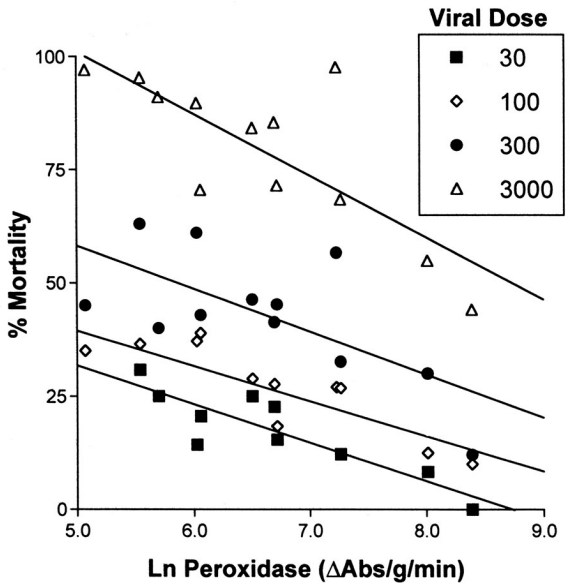

FIG. 2. Influence of POD activity on mortality of *H. virescens* dosed on induced or uninduced cotton foliage with AcMNPV. Each line represents one of four viral doses. Array of POD activities was produced by induction of POD by caterpillar chewing and includes data for both induced and uninduced plants (see Figure 1).

TABLE 2. INFLUENCE OF PHYTOCHEMICALS ON MORTALITY OF *H. virescens* DOSED WITH BACULOVIRUS AcMNPV ON INDUCED OR UNINDUCED COTTON[a]

Variable	Parameter coeff.	Effect on larval mortality	t	P
POD	−0.84	↓	−10.17	<0.0001
Catechols	−0.22	↓	−3.34	=0.0013
Noncatechols[b]	+0.14	↑	+9.48	<0.0001
Viral dose	+1.47	↑	+17.97	<0.0001

[a] Logistic regression model includes both induced and uninduced data. Model chi-square = 598, $df = 4$, $P < 0.0001$; Probability of dying (p) = $\exp(\beta X)/[1 + \exp(\beta X)]$, where $\beta X = [+1.79 - 0.84(\log_e \text{POD}) - 0.22(\text{Catechols}) + 0.14(\text{Noncatechols}) + 1.47(\log_e \text{Viral dose})]$.
[b] Noncatechols = total phenolics − catecholic phenolics.

Influence of Induction of PPO in Tomato on Mortality of H. zea *by HzSNPV.* Chewing damage by larvae of *H. zea* significantly induced PPO activity in damaged tomato leaves an average of fivefold compared with undamaged controls (Figure 3). Chewing damage did not significantly alter POD activity (Figure 3) or plant phenolic levels in four of five replicates. Induction of PPO had no effect on mortality of larval *H. zea* dosed with HzSNPV (Table 3). However, mortality was significantly higher on tomato than on artificial diet, except at the highest viral dose.

Despite the absence of an effect on mortality by the induction of PPO, POD activity of these plants was related to inhibition of viral disease. The higher the POD (Figure 4) or total phenolic levels of damaged or undamaged tomato foliage, the lower the probability of an insect dying from viral infection (Table 4). The higher the catecholic phenolic level, the higher the probability of dying from viral infection (Table 4). Mean catecholic and total phenolic levels in induced vs. uninduced foliage were 1.6 ± 0.3 vs. 1.9 ± 0.4 and 3.6 ± 0.8 vs. 4.0 ± 0.9 μmol/g, respectively (N = 5–6 plants/treatment/replicate). However, catecholic phenolics were two times higher in uninduced foliage in one replicate (1.8 vs. 0.9 μmol/g, $t = 5.0$, $P = 0.0002$).

Confirmation of Influence of Induction of POD in Tomato on Mortality of H. zea *by HzSNPV.* We endeavored to verify whether induction of POD in tomato would significantly alter mortality caused by HzSNPV because induction of PPO in tomato was not correlated with viral disease, but POD activity was as described above. UV damage significantly induced POD activity in damaged tomato an average of 5.5-fold compared with undamaged controls (Figure 5). As a consequence, mortality of *H. zea* larvae was significantly lower on POD-

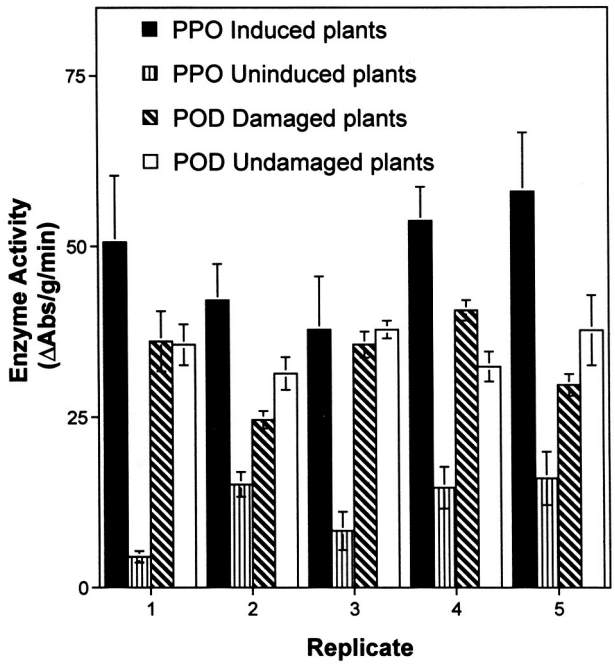

FIG. 3. Induction of PPO in tomato foliage by caterpillar feeding. There were five replicates, done at different times. Bars represent the mean enzyme activities of five to six plants per treatment per replicate. Error bars are the standard error of the mean (some errors too small to portray). Mean PPO activity was significantly higher in damaged foliage within each replicate at the 5% level.

TABLE 3. EFFECT OF INDUCTION OF PPO IN TOMATO ON MORTALITY OF *H. zea* DOSED WITH HzSNPV[a]

	Mortality (%)			
	3 OBS/ larva	7 OBS/ larva	20 OBS/ larva	400 OBS/ larva
PPO-induced tomato	31.7a	50.9a	83.0a	97.7a
Uninduced tomato	32.1a	41.1a	79.7a	100a
Diet	18.9b	31.2b	61.8b	97.7a

[a]OBS = occlusion bodies. Mean percentage mortality of five replicates with 35–45 larvae of *H. zea* per replicate. Means in the same column followed by different letters are significantly different at the 5% level by Fisher's PLSD. ANOVA treatment $F = 10.91$, $df = 2, 48$, $P = 0.0001$; dose $F = 166$, $df = 2, 48$, $P < 0.0001$.

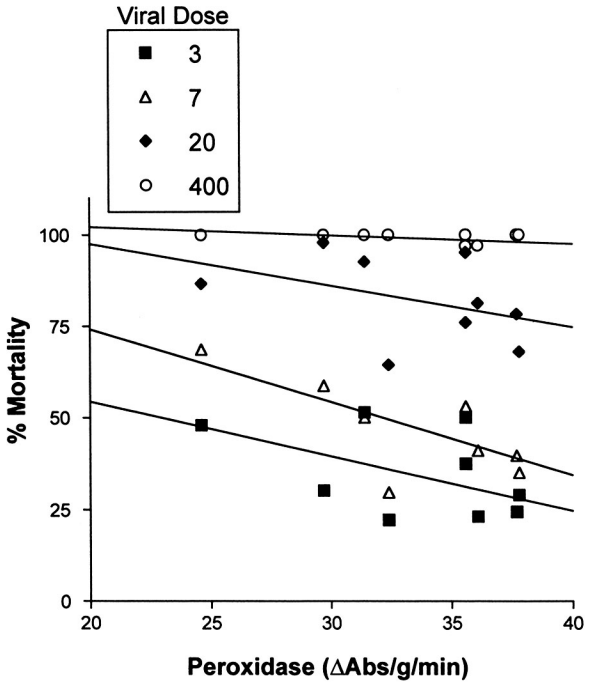

FIG. 4. Influence of POD activity on mortality of *H. zea* dosed with HzSNPV on tomato foliage from PPO-induced (chewing damaged) and uninduced (control) plants (see Figure 3). Each line represents one of four viral doses.

TABLE 4. INFLUENCE OF PHYTOCHEMICALS ON MORTALITY OF *H. zea* DOSED WITH BACULOVIRUS HzSNPV ON PPO-INDUCED OR UNINDUCED TOMATO[a]

Variable	Parameter coeff.	Effect on larval mortality	*t*	*P*
POD	−1.75	↓	−3.66	0.0005
Catechols	+0.42	↑	+2.90	0.0048
Noncatechols[b]	−0.28	↓	−4.00	0.0002
Viral dose	+1.19	↑	+15.97	<0.0001

[a]Logistic regression model includes both induced and uninduced data. Model chi-square = 635, $df = 4$, $P < 0.0001$; probability of dying (p) = $\exp(\beta X)/[1 + \exp(\beta X)]$, where $\beta X = [+4.27 - 1.75(\log_e \text{POD}) + 0.42(\text{Catechols}) - 0.28(\text{Noncatechols}) + 1.19(\log_e \text{Viral dose})]$.

[b]Noncatechols = total phenolics − catecholic phenolics.

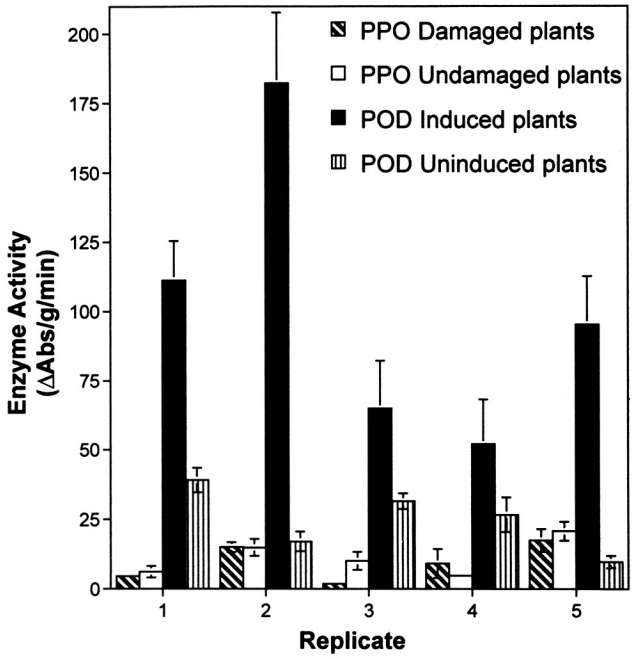

FIG. 5. Induction of POD in tomato foliage by UV irradiation. There were five replicates, done at different times. Bars represent the mean enzyme activities of five to six plants per treatment per replicate. Error bars are the standard error of the mean (some errors too small to portray). Mean POD activity was significantly higher in damaged foliage within each replicate at the 5% level.

TABLE 5. EFFECT OF INDUCTION OF POD IN TOMATO ON MORTALITY OF *H. zea* DOSED WITH HzSNPV[a]

	Mortality (%)		
	3 OBS/larva	7 OBS/larva	400 OBS/larva
POD-induced tomato	14.1a	22.0a	82.5a
Uninduced tomato	25.7b	33.1b	89.0a
Diet	35.8b	45.1b	88.2a

[a]OBS = occlusion bodies. Mean percentage mortality of five replicates with 35–45 larvae of *H. zea* per replicate. Means in the same column followed by different letters are significantly different at the 5% level by Fisher's PLSD. ANOVA treatment $F = 5.31$, $df = 2, 36$, $P = 0.0095$; dose $F = 82.4$, $df = 2, 36$, $P < 0.0001$.

induced than on uninduced foliage at the two lowest viral doses (Table 5). The average reduction in mortality on irradiated versus nonirradiated foliage was 45% at the LD_{25}, 34% at the LD_{50}, and 7% (not significant) at the LD_{99} (Table 5). Mortality also was significantly lower when insects consumed virus on induced tomato compared with diet controls. Mortality was equivalent whether larvae consumed virus on uninduced tomato or artificial diet. UV damage did not significantly alter PPO activity (Figure 5).

Contrary to the above results, there was one replicate in which larval mortality was lower on the uninduced foliage. Although UV damage did not alter phenolic levels in four of five replicates (N = 5–6 plants/treatment/replicate), in one replicate catecholic and total phenolics were three times and 1.7 times higher, respectively, in uninduced vs. induced foliage (5.7 ± 0.8 vs. 1.9 ± 0.3 and 4.2 ± 0.5 vs. 2.8 ± 0.3 μmoles/g, respectively; t = 4.5, P = 0.0008 and t = 2.4, P = 0.0324, respectively). In this replicate, percent mortalities were 23 vs. 8.9% at 3 OBS/larva, 28 vs. 20% at 7 OBS/larva, and 91 vs. 89% at 400 OBS/larva on induced vs. uninduced foliage, respectively.

Across all replicates, the higher the POD activity (Figure 6) or phenolic

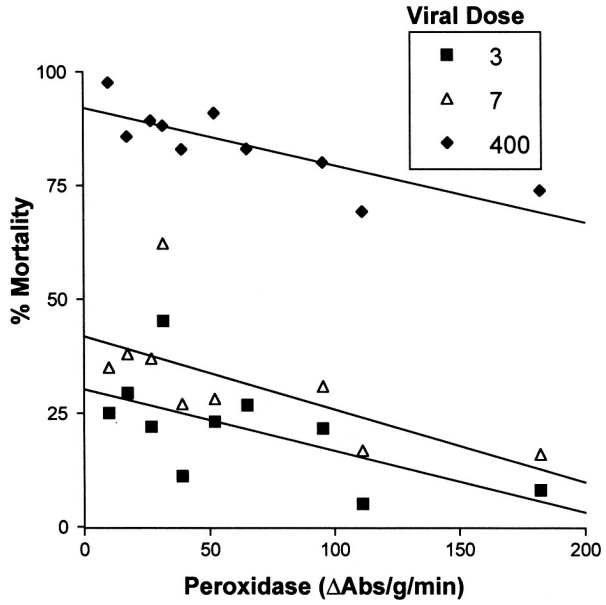

FIG. 6. Influence of POD activity on mortality of *H. zea* dosed with HzSNPV on tomato foliage from POD-induced (UV damaged) and uninduced (control) plants (see Figure 5). Each line represents one of three viral doses.

TABLE 6. INFLUENCE OF PHYTOCHEMICALS ON MORTALITY OF *H. zea* DOSED WITH BACULOVIRUS HzSNPV ON POD-INDUCED OR UNINDUCED TOMATO[a]

Variable	Parameter coeff.	Effect on larval mortality	t	P
POD	−0.26	↓	−2.99	0.0042
Total phenolics	−0.12	↓	−2.87	0.0057
Viral dose	+0.68	↑	+17.92	<0.0001
Catechols[1]	−0.56	↓	−2.65	0.0103

[a]Logistic regression model includes both induced and uninduced data. Two equally significant models were obtained by logistic regression: model 1: probability of dying (p) = $\exp(\beta X)/[1 + \exp(\beta X)]$, where $\beta X = [-0.88 - 0.26(\log_e \text{POD}) - 0.12(\text{Total phenolics}) + 0.68(\log_e \text{Viral dose})]$; chi-square = 467, $df = 3$, $P < 0.0001$. model 2[1]: $\beta X = [+0.21 - 0.68(\log_e \text{POD}) - 0.57(\text{Catechols})^2 + 0.68(\log_e \text{Viral dose})]$; chi-square = 466, $df = 3$, $P < 0.0001$.

content of induced or uninduced tomato foliage, the lower the probability of an insect dying from viral infection (Table 6).

DISCUSSION

We used the differences in type and degree of inducibility of PPO and POD in cotton and tomato to garner further evidence that POD and phenolic substrates are causal agents in the inhibition of baculoviral disease on some host plants. In both cotton and tomato, induction of POD was negatively correlated with the ability of two different baculoviruses to kill their noctuid hosts (see Figure 7 for a schematic representation of the models). Thus, we now have substantial regressional evidence in two different plant–insect–virus systems that plant POD activity may protect insects from disease caused by baculoviruses. These data support our previous finding that viral efficacy could be predicted from POD activity among different plant species in a dose-dependent manner (Hoover et al., 1998). We should point out that, in addition to POD, UV irradiation also induces jasmonate-sensitive compounds in tomato such as proteinase inhibitors (PIs) (Conconi et al., 1996). However, preliminary studies using diet incorporation suggest that PIs mildly enhance mortality by baculovirus (Hoover et al., unpublished).

In contrast to induction of POD, induction of another plant oxidative enzyme, PPO, had no effect on viral activity on tomato. This finding is in contrast to a previous study showing that oxidation of phenolics in homogenized tomato foliage by PPO decreased mortality in *H. zea* by baculovirus (Felton and Duffey, 1990). The difference between our findings may be due to Felton

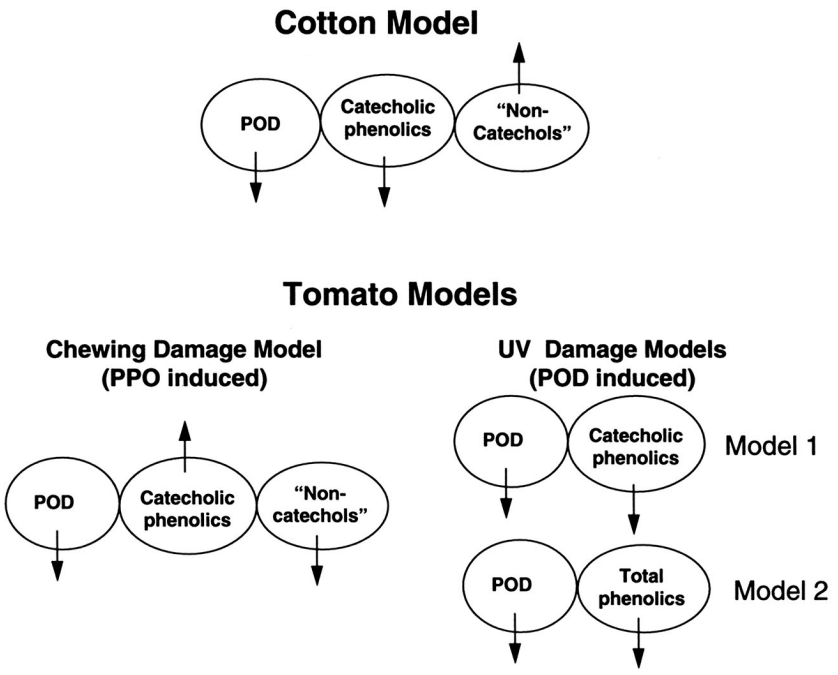

FIG. 7. Schematic representation of the influence of phytochemical induction on mortality of noctuid larvae treated with baculoviruses. Chemical with downward arrow indicates decreased mortality by baculovirus as chemical level increased. Chemical with upward arrow indicates increased mortality by baculovirus as chemical level increased. All models share in common the inhibition of baculoviral disease by POD.

and Duffey's (1990) use of an *in vitro* rather than an *in vivo* assessment of the impact of this enzyme on viral activity. Alternatively, this difference may be due to differences in the isozyme(s) of PPO that are induced. PPO is present in both chloroplasts and trichomes, and it is not known if both are inducible.

There are a number of possible explanations for the difference between PPO and POD in their influence on viral disease. Despite the fact that both POD and PPO catalyze phenolic oxidation, the type and mechanism of end-product formation is different for catalysis by POD than PPO. POD catalyzes oxidation of a wider range of substrates, producing a number of free radical intermediates in the process, such as semiquinones, active oxygen species, H_2O_2, and other organic radicals (Butt, 1981; Butt and Lamb, 1981; Robinson, 1991; Ahmad, 1995). We propose that free radical generation may be the most significant process leading to inhibition of viral disease. PPO, in contrast, directly

produces the quinone using O_2 as a cosubstrate (Mayer, 1987). PPO activity may be much less detrimental to the virus because it is less effective at secondarily producing free radicals by redox cycling. Free radicals are known to be highly destructive, especially in their interaction with macromolecules such as proteins, lipids, and DNA (Felton et al., 1994; Summers and Felton, 1994; Ahmad, 1995; Felton, 1995). The action of organic free radicals and/or active oxygen species on the proteinaceous occlusion body and/or envelope of the virions of the baculovirus may be responsible for the observed inhibition of viral disease. Inhibition of viral disease by reactions catalyzed by POD and not PPO may also be related to the overall availability of H_2O_2 vs. O_2 in the insect's digestive system. Whatever the mechanism, baculoviruses appear to be more sensitive to the products of POD.

Different classes of phenolic substrates were correlated with different impacts on the lethality of baculoviruses depending upon which enzyme was induced or which plant was examined. Differences in these relationships may simply be the result of covariance between the independent variables of induced enzyme and phenolic group. However, these differences may be biologically based. Phenolics, particularly some of the catecholic phenolics, have a variety of reactivities. In a basic environment and/or in the presence of PPO or POD, these reactivities include radical scavenging, reducing power, chelation, inhibition of phenolases and other metal-containing oxidative enzymes, and prooxidative activity (initiation of chain reactions generating active oxygen species, organic free radicals, and/or quinones) (Golan-Goldhirsh and Whitaker, 1984; Fazal et al., 1990; Huang et al., 1992; McEvily et al., 1992; Ahmad, 1995). The degree and proportion to which they exhibit these reactivities is dependent upon the specific type of phenolic, relative concentrations, and chemical context. For example, although quercetin usually acts as a prooxidant (Huang et al., 1992; Ahmad, 1995), it can also scavenge superoxide anions, as can rutin and other flavanoids (Robak and Gryglewski, 1988). In addition, catecholic phenolics can, either by enzymatic or autooxidation mechanisms, generate superoxide free radical ions, H_2O_2, and hydroxyl radicals that can lead to a variety of reactions with protein directly and/or with other organic materials (Huang et al., 1992; Summers and Felton, 1994; Ahmad, 1995; Felton, 1995). The generation of such reaction products is known to be highly toxic to insects by a variety of mechanisms (Summers and Felton, 1994; Ahmad, 1995; Bi and Felton, 1995; Felton, 1995). As a consequence, these reaction products may attenuate viral disease by binding to viral proteins preventing infection from occurring, and/or by damaging infected midgut cells, leading to sloughing of the infected cells before the virus becomes established. In another study, hydroperoxides, an indicator of oxidative stress, were significantly higher in the midgut of larval *H. zea* that fed on damaged cotton plants compared to larvae that consumed virus on undamaged plants (Bi and Felton, 1995). Thus, at least on cotton,

oxidative damage to the insect midgut may be a mechanism of inhibition of viral disease in insects that consume virus on damaged foliage.

It is also possible that some of the relationship between phenolics and disease may be obscured by limitations in our ability to measure insoluble phenolics bound to the cell wall in aqueous methanol extracts. It may be that when POD and/or PPO are induced, there is also an increase in insoluble phenolics bound to the cell wall, limiting our ability to evaluate these relationships realistically.

Although our major aim was to verify a hypothetical mechanism whereby plant phenolase activity inhibits disease by baculoviruses, our finding that induced "plant defenses" can impair the ability of a natural enemy to control pest insects raises questions about the ecological consequences of these induced defenses in a tritrophic system. The ecological consequences of induction on each organism in a tritrophic system depend upon a balance between positive and negative forces and whether the third trophic level is occupied by a pathogen or a parasitoid (Campbell and Duffey, 1979, 1981; Felton et al., 1987; Felton and Duffey, 1990; Hunter and Schultz, 1993; Hoover et al., 1998). For example, in the work by Hunter and Schultz (1993), gypsy moth larvae were less susceptible to viral infection by *Lymantria dispar* NPV consumed on foliage from branches that had been previously defoliated. Although high levels of oak tannins lead to deleterious effects on gypsy moth populations, induction of gallotannins may be detrimental to the plant by partially protecting gypsy moth larvae from their (LdMNPV) enemy. Likewise, induction of oxidative enzymes in response to herbivory is generally thought to benefit plants (Duffey and Felton, 1989, 1991; Bi et al., 1997). However, when natural enemies of the herbivore can be inhibited by the induction of "plant defensive compounds," previous herbivory may benefit the herbivore more than the plant. Consequently, whether plant oxidative activity is induced or constitutive, the ability of secondary plant compounds to inhibit baculoviral disease may render bacterial diseases incompatible with host plant resistance in integrated pest management programs (Felton et al., 1987; Felton and Duffey, 1990; Hunter and Schultz, 1993; Hoover et al., 1997). However, whether our findings that plant phenolase inhibits baculoviral disease in noctuid larvae can be extrapolated from the laboratory to the field, where there are even more interactive factors, remains to be investigated.

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ENANTIOMERIC COMPOSITION OF MONOTERPENE HYDROCARBONS IN SOME CONIFERS AND RECEPTOR NEURON DISCRIMINATION OF α-PINENE AND LIMONENE ENANTIOMERS IN THE PINE WEEVIL, *Hylobius abietis*

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Abstract—The enantiomeric composition of seven monoterpene hydrocarbons in headspace volatiles of spruce sawdust and seedlings (*Picea abies*), pine seedlings (*Pinus sylvestris*), and branches of juniper (*Juniperus communis*) was determined by gas chromatographic separation on a β-Cyclodextrin column. For the six monoterpenes, α-pinene, camphene, β-pinene, sabinene, limonene, and β-phellandrene, both enantiomers were present, whereas for 3-carene only the (+)-configuration was found. The amount of each enantiomer varied considerably both in relation to total amount of all of them, and for the six pairs also in relation to the opposite enantiomer. One olfactory receptor neuron in the pine weevil (*Hylobius abietis*) showed a strong response to α-pinene when stimulated with all four headspace materials via a GC equipped with a DB-WAX column. The same neuron was subsequently tested with repeated stimulations via the GC effluent containing the (+)- or (−)-enantiomer. A marked better response to (+)- than to (−)-α-pinene was elicited. Another olfactory receptor neuron that responded strongly to limonene when stimulated with the spruce volatiles was tested for enantiomers of limonene. This neuron responded more strongly to (−)- than to (+)-limonene, when stimulated alternately with each of the limonene enantiomers. Discrim-

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ination between enantiomers by plant olfactory receptor neurons suggests that the enantiomeric ratios of volatile compounds may be important in host location by the pine weevil.

Key Words—*Hylobius abietis*, plant odors, conifers, monoterpene enantiomers, olfaction, receptor neuron responses, gas chromatography–electrophysiology.

INTRODUCTION

The steriochemistry of volatile compounds is important in many signal–receptor interactions, and attention has been paid to discrimination of enantiomers of chiral compounds in olfaction. Of particular interest in this respect are insect pheromones, since they are known olfactory signals for which specific receptor neurons have evolved (c.f., Silverstein, 1979). Receptor neuron discrimination between enantiomers of pheromones has been studied in bark beetles (Mustaparta, 1979, 1980; Mustaparta et al., 1980; Wadhams et al., 1982) and in a moth species (Hansen et al., 1983). These results demonstrated that there is one type of receptor neuron specialized for each, or sometimes for only one, of the enantiomers tested in the various species. Furthermore, in tests of single compounds and racemates, no interaction seems to take place between enantiomers at the receptor neurons (Mustaparta et al., 1980). This means that olfactory information about different pheromone enantiomers is received by separate receptor neurons.

Plant odors also may contain chiral components. Most plants produce a variety of volatile compounds characteristic of each species. However, large individual variations are found, as in spruce (*Picea abies*), in which two different genotypes produce different ratios of the monoterpenes limonene and myrcene (Schönwitz et al., 1989). Several chemotypes of pine (*Pinus sylvestris*) have been identified, some exhibiting high levels of 3-carene and others with high limonene content (Yazdani et al., 1985). Large differences of enantiomeric composition also occur between individual trees as well as between tissues of one tree (Borg-Karlson et al., 1993; Persson et al., 1993, 1996). Bark beetles that utilize plant volatiles as precursors for pheromone production may convert only one enantiomer to a pheromone. For example, (−)-α-pinene is converted by *Ips paraconfusus* and *I. typographus* to the pheromone component (+)-*cis*-verbenol (Renwick et al., 1976; Byers, 1981; Lindström et al., 1989).

The pine weevil, *Hylobius abietis*, is highly attracted to volatiles produced by seedlings of pine (*Pinus sylvestris*) and spruce (*Piece abies*), and is slightly less attracted to juniper (*Juniperus communis*), which is regarded as a nonhost to *H. abietis* (Nordlander, personal communication). The volatile profile of these three plant species contains many of the same compounds, and monoterpene hydrocarbons are often the major constituents (Wibe et al., 1997). Many of

these compounds elicited responses from receptor neurons in the pine weevil when the neurons were stimulated directly with synthetic compounds (Mustaparta, 1975), and with GC-separated headspace volatiles as they exited a column with the effluent split between the detector and an exit port (Wibe and Mustaparta, 1996; Wibe et al., 1997). Wibe et al. (1997) focused upon how receptor neurons discriminated between compounds of natural plant volatile blends and provided the basis for classification of the receptor neurons into 30 types. Two of these neuron types contained receptor neurons specialized for different enantiomers of α-pinene and limonene, since they showed a different ranking of response strengths to other analogs (Wibe et al., 1997). In the present paper we present data on enantiomeric compositions of seven volatile monoterpenes, including α-pinene and limonene, from three conifer species. In addition, two receptor neurons, one specialized for α-pinene and one for limonene, are shown to discriminate between the enantiomers of the two compounds.

METHODS AND MATERIALS

Insects. Pine weevils (*Hylobius abietis*) were collected during the swarming season at a sawmill near Trondheim, Norway. These were prepared for electrophysiological studies as described by Wibe and Mustaparta (1996).

Volatiles. Volatiles released from the following plant materials were used for stimulating receptor neurons of the pine weevil: 100 seedlings of spruce (*Picea abies*), 150 liters of spruce sawdust, 100 seedlings of pine (*Pinus sylvestris*), and branches of juniper (*Juniperus communis*) with the amount of the material about the same as for the seedlings. These were the same samples used in a previous study by Wibe and Mustaparta (1996) and were collected by drawing air from the plant material through Porapak Q. The monoterpenes α-pinene, camphene, β-pinene, sabinene, 3-carene, limonene, and β-phellandrene, previously identified by gas chromatography–mass spectrometry (GC-MS) (Wibe et al., 1997), were analyzed with respect to enantiomeric composition. Samples of enantiomers of limonene and α-pinene were obtained from Aldrich. Enantiomeric purities were: (+)-α-pinene 99.1%, (−)-α-pinene 95.4%, (+)-limonene 99.7%, (−)-limonene ~90%.

Separation of Enantiomers. For analyzing the enantiomeric composition of fractions of the volatile mixtures, a two-dimensional GC system was used as described by Borg-Karlson et al. (1993). Two GCs were connected with a heated interface equipped with time-programmed pneumatic microvalves. The first GC had a DB-Wax capillary column for separating the components of the mixtures. Selected fractions of the mixtures were transferred via the valve into the second GC equipped with a β-Cyclodextrin fused silica column, which separated the

enantiomers of the seven monoterpenes. A more detailed description of the gas chromatographic procedure and temperature programming is found in Persson et al. (1996).

Single Cell Recordings and Stimulation. Nerve impulses from single olfactory receptor neurons located on the antennal club of the pine weevil were recorded extracellularly with tungsten microelectrodes as described by Wibe and Mustaparta (1996). Receptor neurons responding to the vapor of the headspace-collected volatiles in a syringe olfactometer (Kafka, 1970; Mustaparta et al., 1980) were further examined by stimulating the neuron with the separated components exiting from a gas chromatograph. After separation of the volatile compounds in the GC column, half of the effluent was led to the GC detector and the other half passed out of the GC oven to the insect antenna. Thus, the response of the single receptor neurons to each component was recorded simultaneously with the gas chromatogram.

RESULTS

Enantiomeric Composition of Monoterpenes in Plant Volatiles. The enantiomers of α-pinene, camphene, β-pinene, sabinene, 3-carene, limonene, and β-phellandrene were separated and identified in spruce sawdust, spruce seedlings, pine seedlings, and juniper branches. The molecular structures of the enantiomers are shown in Figure 1. The amount of each enantiomer varied considerably among plant materials both in relation to the total amount of all seven monoterpenes (Table 1) as well as in relation to the opposite enantiomer (enantiomeric ratio) (Table 2). Whereas all four plant materials contained both enantiomers of α-pinene as major constituents (10.3–29.0%), a marked difference was found for several other compounds (Table 1). (−)-β-Pinene, (−)-

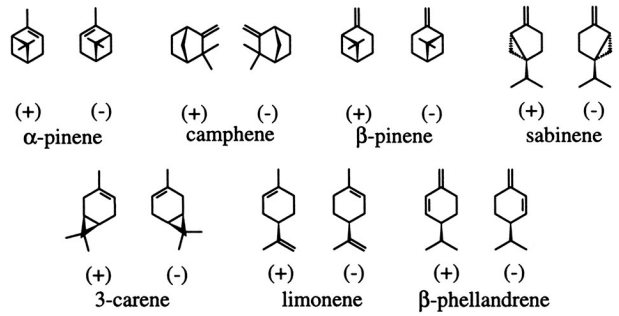


FIG. 1. The molecular structure of the enantiomeric pairs of the seven chiral monoterpene hydrocarbons studied.

TABLE 1. RELATIVE AMOUNTS OF SEVEN ENANTIOMERIC PAIRS OF MONOTERPENE HYDROCARBONS IN HEADSPACE VOLATILES OF SPRUCE SAWDUST, SPRUCE SEEDLINGS, PINE SEEDLINGS, AND JUNIPER BRANCHES[a]

Compound	Spruce sawdust	Spruce seedling	Pine seedling	Juniper branches
(+)-α-Pinene	22.4	10.3	29.0	18.0
(−)-α-Pinene	18.9	27.5	17.6	18.1
(+)-Camphene	0.3	1.9	0.5	0.1
(−)-Camphene	0.9	7.7	0.8	0.2
(+)-β-Pinene	0.8	0.4	0.4	0.5
(−)-β-Pinene	20.7	18.6	2.2	1.3
(+)-Sabinene	0.1	1.0	<0.05	47.4
(−)-Sabinene	<0.05	1.8	1.8	0.1
(+)-3-Carene	5.1	0.4	45.8	4.7
(−)-3-Carene	0	0	0	0
(+)-Limonene	3.9	2.5	0.3	5.7
(−)-Limonene	16.7	13.0	0.8	0.5
(+)-β-Phellandrene	0.7	0.4	<0.05	0.3
(−)-β-phellandrene	9.5	14.7	0.8	2.8

[a]The values are expressed as percentages of the total amounts of the seven pairs of monoterpenes according to the GC.

limonene and (−)-β-phellandrene, were major components (9.5–20.7%) in spruce and minor components (0.5–2.8%) in pine and juniper. Pine contained a large amount of (+)-3-carene (45.8%) and juniper a large amount of (+)-sabinene (47.4%) in addition to the α-pinene enantiomers.

All plant materials contained both enantiomers of six of the monoterpenes, but only the (+)-configuration of 3-carene (Table 1). For the six monoterpenes, the enantiomeric ratio varied between them as well as between plant materials (Table 2). The highest enantiomeric purity was found for (+)-sabinene (99.8%) in juniper, (−)-sabinene (97.8%), and (−)-β-phellandrene (99.3%) in pine seedlings and for (−)-β-pinene (96.2% and 97.9%) in spruce materials. The largest variations of enantiomeric ratios between plant materials were found for sabinene and limonene. The (+)-enantiomers were by far the greater part in juniper, whereas in spruce and pine seedlings the (−)-enantiomers were present in larger amounts than the (+)-configurations. Thus, the ratio between (+)- and (−)-sabinene differed from about 500:1 (99.8:0.2) in juniper to 1:44 (2.2:97.8) in pine, and for the limonene enantiomers from about 12:1 (92.5:7.5) in juniper

to about 1:4 (19.0:81.0) in spruce. For α-pinene and camphene, the ratio between the (+)- and the (−)-enantiomers varied from approximately 1:1 (54.3:45.7) to 1:4 (19.7:80.3) in all materials (Table 2).

Receptor Neuron Responses to Enantiomers. Two receptor neurons, classified as an α-pinene (neuron 1) and a limonene (neuron 2) type, were tested for response to the enantiomers of their key compounds.

The classification of the α-pinene receptor neuron (neuron 1) was based on stimulation via the GC split of components from headspace volatiles of spruce sawdust, spruce seedlings, pine seedlings, and juniper branches. All recordings showed a strong response to the α-pinene peak of the four samples, but only the chromatogram of spruce sawdust is shown in Figure 2. The neuron also responded weakly to other compounds, of which the most significant were camphor, β-pinene, and pinocamphone (Figure 2; peaks 7, 2, and 8, respectively). When stimulating the neuron with 1 ng (+)- and (−)-α-pinene in the GC effluent, a clear discrimination between the two enantiomers was shown. During seven stimulations, alternating between the (+)- and the (−)-enantiomer and ending with the racemate, significantly stronger responses were recorded to the

TABLE 2. ENANTIOMERIC COMPOSITION (%) OF SEVEN MONOTERPENES IDENTIFIED IN HEADSPACE VOLATILES OF SPRUCE SAWDUST, SPRUCE SEEDLINGS, PINE SEEDLINGS, AND JUNIPER BRANCHES

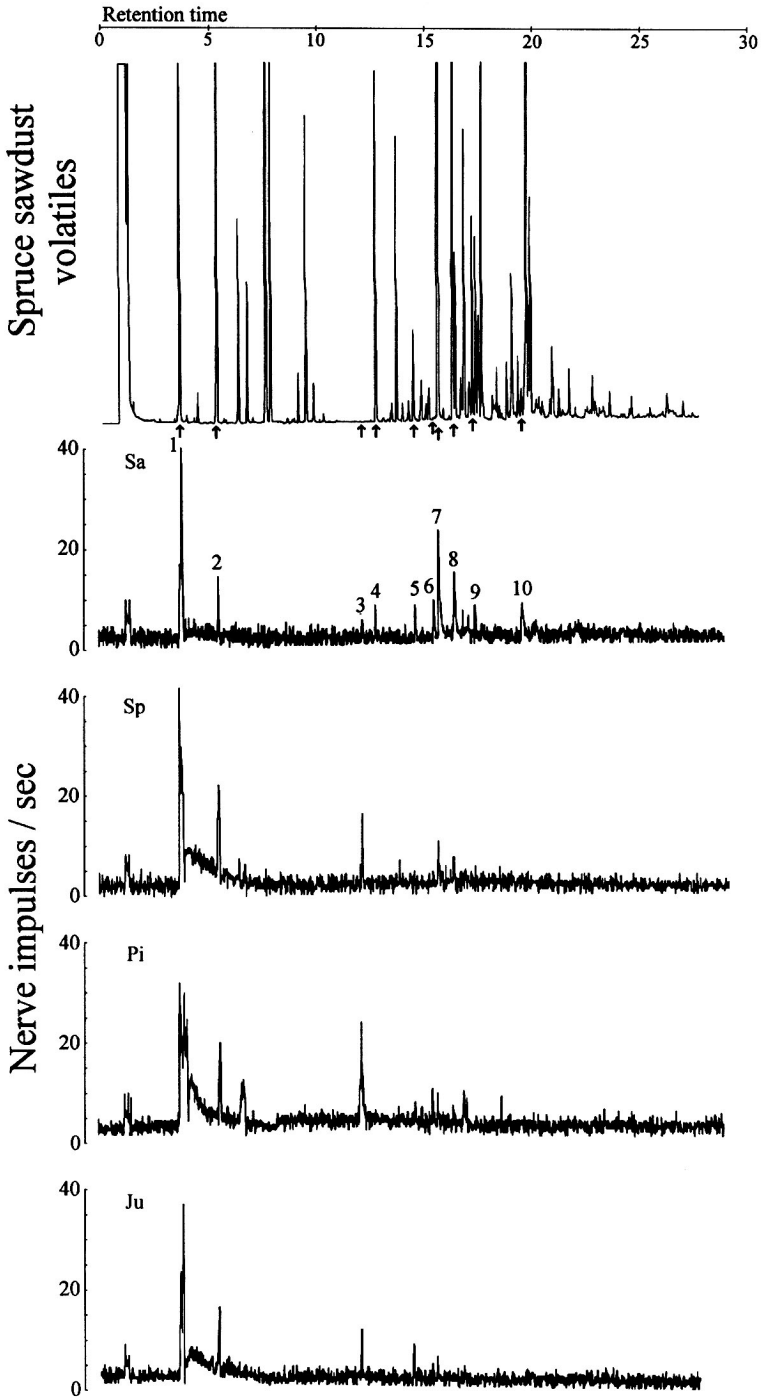
Compound	Spruce sawdust	Spruce seedling	Pine seedling	Juniper branches
(+)-α-Pinene	54.3	27.2	62.2	49.9
(−)-α-Pinene	45.7	72.8	37.8	50.1
(+)-Camphene	25.8	19.7	38.2	38.1
(−)-Camphene	74.2	80.3	61.8	61.9
(+)-β-Pinene	3.8	2.1	16.7	28.0
(−)-β-Pinene	96.2	97.9	83.3	72.0
(+)-Sabinene	85.7	35.4	2.2	99.8
(−)-Sabinene	14.3	64.6	97.8	0.2
(+)-3-Carene	100.0	100.0	100.0	100.0
(−)-3-Carene	0	0	0	0
(+)-Limonene	19.0	16.0	29.5	92.5
(−)-Limonene	81.0	84.0	70.5	7.5
(+)-β-Phellandrene	6.4	2.5	0.7	9.8
(−)-β-Phellandrene	93.6	97.5	99.3	90.2

(+)- than to the (−)-enantiomer and an intermediate response was recorded to the racemate (Figure 3). The responses lasted 3–4 sec, and the number of spikes during 3 sec of the highest firing frequency period are presented in Table 3. A marked and consistently higher firing rate in response to the (+)-enantiomer (average 99 impulses sec) than to the (−)-enantiomer (average 35 impulses sec) is shown. The intermediate response to the racemate, tested once, was 81 impulses/sec.

The other receptor neuron (neuron 2) responded strongly to limonene when tested with spruce sawdust and spruce seedling volatiles (Figure 4; peak 4). Up to 10 additional weaker responses were recorded, and the three largest responses were elicited by γ-terpinene, sabinene, and (+)-3-carene (Figure 4; peaks 6, 2, and 3, respectively). In addition, solvent strongly activated this neuron. The response to the large amount of limonene was actually stronger than recorded in peak 4, since the extracellularly recorded spike amplitudes diminished as the frequency increased, falling under the threshold level of the electronic recording system. A smaller amount ($< 100\times$) was used for the subsequent testing of the enantiomeric effects. When the neuron was stimulated via the GC effluent with 1 ng each of (+)- and (−)-limonene, different frequencies were recorded in response to the two enantiomers (Figure 5). By alternating stimulations with (+)- and (−)-limonene, a consistently stronger response to the (−)-enantiomer than to the (+)-enantiomer was obtained. The average number of spikes during the highest response frequency was 78 impulses/sec for (−)-limonene as compared to 61 impulses/sec for (+)-limonene (Table 3). The response to the racemate tested at the end of the experiment showed an intermediate response (65 impulses/sec), closer to the less effective enantiomer.

DISCUSSION

The finding that receptor neurons discriminate between different plant volatiles suggests that the enantiomers may be used by the pine weevil to detect host and nonhost plant species. The present results support the suggestion made previously that the α-pinene and the limonene neurons of the pine weevil are tuned to one of the enantiomers, which was based on the different ranking of response strengths of analog compounds (Wibe et al., 1997). Although the present data are very limited and show discrimination of enantiomers by only two neurons, the tests of six repeats demonstrate a consistent difference between the responses to each enantiomer with one neuron responding better to (+)- than to (−)-α-pinene and the other better to (−)- than to (+)-limonene. Whether there are, in addition, other receptor neurons specialized for (−)-α-pinene and (+)-limonene remains to be investigated by testing pure enantiomers. The tests


Retention time
0
5
10
15
20
25
30
Spruce sawdust volatiles
1
2
3
4
5
6
7
8
9
10
Nerve impulses / sec
Sa
Sp
Pi
Ju
40
20
0

with the racemates, showing intermediate responses, suggest that no interaction between the enantiomers takes place at the receptor neuron. The response difference was larger between (+)- and (−)-α-pinene than between the limonene enantiomers, which might be due to differences of enantiomeric purities, or the membrane receptors for α-pinene might have a higher enantiomeric specificity than the limonene receptors. The latter seems more likely, since the enantiomeric purity of (+)-limonene was higher (99.7%) than of (−)-α-pinene (95.4%). A more detailed study of the dose–response relationship for pure enantiomers is necessary in order to draw definite conclusions about the efficiency of the enantiomeric discriminations.

Pine weevils also may utilize the differential enantiomeric composition of the other monoterpenes in plant odor discrimination, although previous recordings from single receptor neurons have not demonstrated response differences to the GC peaks of the six monoterpenes (Wibe and Mustaparta, 1996). Although predominantly one enantiomer of a compound is present, there might be enough of the less prevalent enantiomer to elicit a strong response. This may explain why stimulation with different plant materials did not show very different responses to corresponding GC peaks, although they contained different ratios of the enantiomers (Wibe et al., 1997).

The enantiomeric composition, determined for monoterpenes released by 100 seedlings of spruce and pine does not give information about individual seedling differences. Variations among individuals and tissues have been studied with respect to enantiomeric composition of all major monoterpenes in *Picea abies* and *Pinus sylvestris* (Persson et al., 1996; Sjödin et al., unpublished). In spite of individual variations, each plant species contains consistently more of one enantiomer than of the other, e.g., *P. sylvestris* contains more (−)- than (+)-α-pinene and vice versa for *P. abies*. The results are in accordance with the enantiomeric ratios of the monoterpenes presented in the present study.

Of particular interest might be the enantiomeric differences between the host and the nonhost plant materials and specifically in this case the presence of (+)-sabinene. No receptor neuron has yet been recorded that responded most strongly to sabinene. The only neuron that responded to sabinene was the limo-

FIG. 2. Simultaneously recorded gas chromatogram of spruce sawdust (above) and single receptor neuron activity during stimulation via the GC effluent with the separated compounds (Sa). Responses to GC separated volatiles of spruce seedlings (Sp), pine seedlings (Pi), and juniper branches (Ju) are also shown. All test series resulted in a strong response to α-pinene (peak 1). During stimulation with the spruce sawdust volatiles, weaker responses were recorded to β-pinene (2), an unidentified compound (3), fenchone (4), α-copaene (5), an unidentified compound (6), camphor (7), pinocamphone (8), fenchol (9), and an unidentified compound (10). When stimulated with the other volatile mixtures, weaker responses also were recorded.

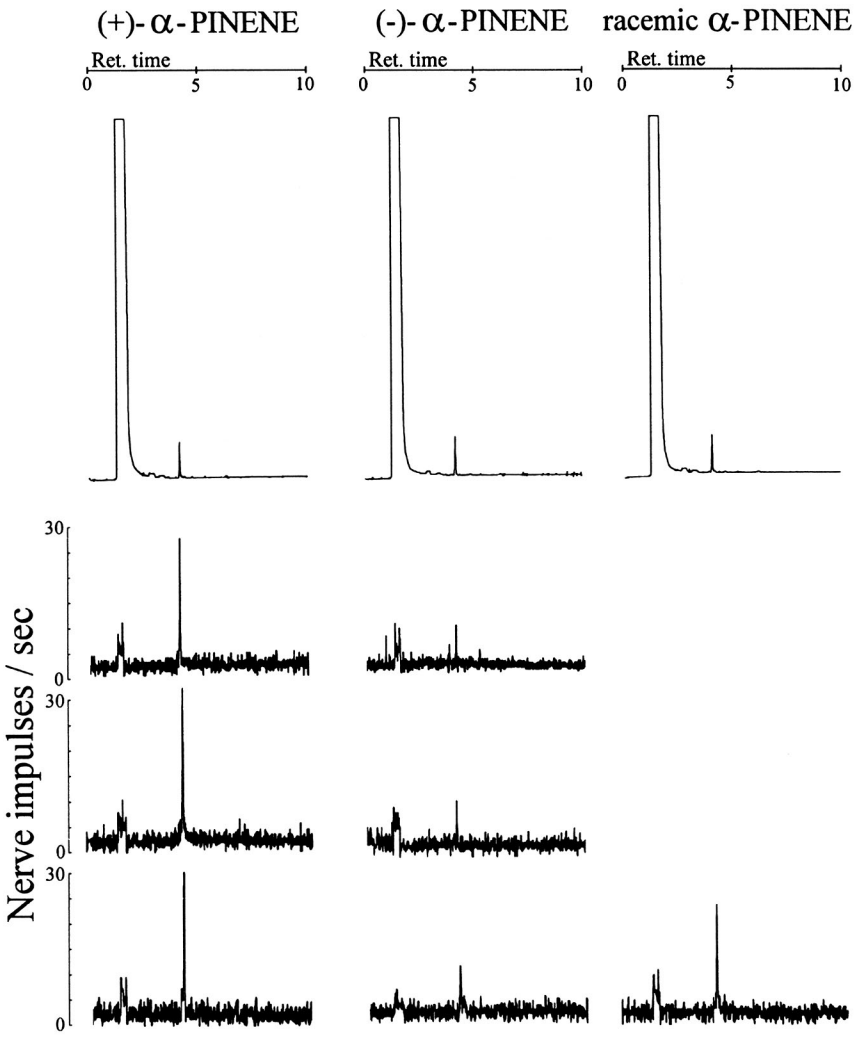


FIG. 3. Gas chromatograms of 1 ng each of (+)-, (−)-, and racemic α-pinene (above), and activity in a single receptor neuron recorded during seven stimulations via the GC effluent, alternating between the enantiomers and ending with the racemate. Strong responses were recorded to (+)-α-pinene, while (−)-α-pinene elicited weaker responses. An intermediate response was recorded to racemic α-pinene.

TABLE 3. NUMBER OF NERVE IMPULSES RECORDED FROM TWO DIFFERENT OLFACTORY RECEPTOR NEURONS OF PINE WEEVIL DURING STIMULATION WITH DIFFERENT ENANTIOMERS OF α-PINENE (NEURON 1) AND LIMONENE (NEURON 2) FROM GC EFFLUENT

Compound	No. of nerve impulses during a 3-sec period			Average no.
Neuron 1				
(+)-α-Pinene	92	94	111	99
(−)-α-Pinene	32	35	39	35
Rac. α-Pinene	81			81
Neuron 2				
(+)-Limonene	60	67	57	61
(−)-Limonene	77	73	83	78
Rac. limonene	65			65

nene neuron presented in Figure 4, and it showed a relatively weak response to sabinene when tested for spruce seedling volatiles containing sabinene as a minor component. Unfortunately, this neuron was not tested for juniper volatiles that contained 100 times more (+)-sabinene than (−)-limonene. Since it was shown that limonene reduce the attraction of the pine weevil to α-pinene and ethanol (Nordlander, 1990, 1991), it is possible that (+)-sabinene might inhibit the attraction of the pine weevil by activating the (−)-limonene neuron. In some insect species receptor neurons for interspecific signals that mediate inhibition of pheromone attraction also mediate information about more than one interspecific signal (Lucas and Renou, 1989; Berg and Mustaparta, 1995).

Another difference is found for the two host materials, spruce and pine. Whereas the major components of the pine material were (+)- and (−)-α-pinene and (+)-3-carene, the spruce materials contained an additional three other major components, (−)-β-pinene, (−)-limonene, and (−)-β-phellandrene. Pine is more attractive than spruce to the pine weevil (Christiansen, 1971). This may be due in part to the higher content of (−)-limonene in spruce (Table 1), which might reduce the attraction elicited by other monoterpenes. Thus, the attraction to spruce may be modulated in part by the ratio of α-pinene and limonene present in individual spruce materials, influencing the ratio of activities in the α-pinene and limonene neurons.

The enantiomers of the seven monoterpenes presented here are only a limited number of the volatiles released from the plants, and they are about one third of the compounds that have been shown to elicit the strongest responses

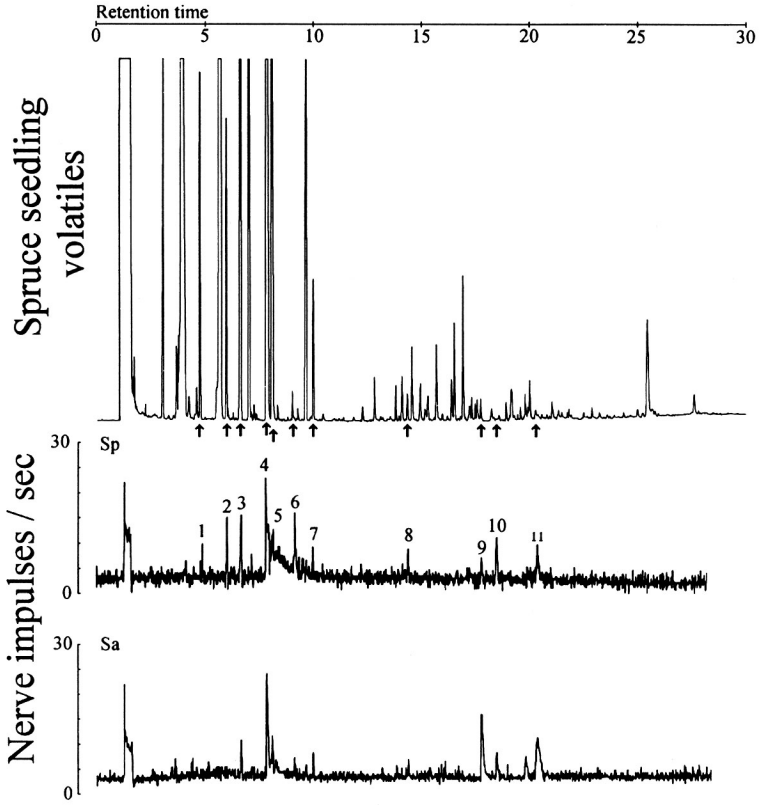


FIG. 4. A simultaneously recorded gas chromatogram of spruce seedlings (above) and single receptor neuron activity during stimulation via the GC effluent containing the separated compounds (Sp). Responses to GC-separated volatiles of spruce sawdust (Sa) are also shown. Both test series resulted in a strong response to the compound identified as limonene (peak 4). During the stimulation with the seedling volatiles, weaker responses were recorded to camphene (1), sabinene (2), (+)-3-carene (3), β-phellandrene (5), γ-terpinene (6), terpinolene (7), α-cubebene (8), terpinene-4-ol (9), an unidentified compound (10), and α-muurolene (11). When stimulated with the spruce sawdust volatiles, weaker responses also were recorded to other compounds.

of the pine weevil receptor neurons. Therefore, it is possible that enantiomeric ratios of many other compounds also may be important for the pine weevil in discriminating host and nonhost odors. Further studies along this line are necessary to get more complete information about the enantiomeric composition of the odor blend or fingerprint used by the pine weevil to locate host and avoid non-host plants.

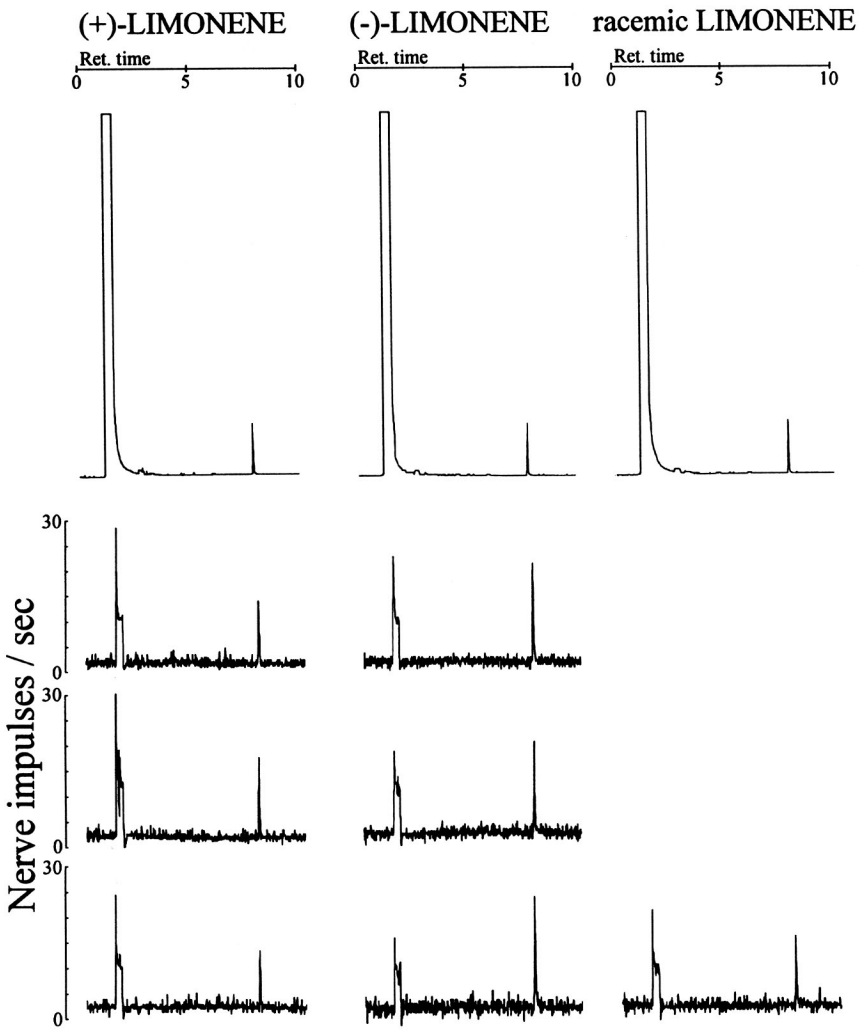


FIG. 5. Gas chromatograms of 1 ng each of (+)-, (−)-, and racemic limonene (above), and activity in a single receptor neuron recorded during seven stimulations via the GC effluent, alternating between the enantiomers and ending with the racemate. Strong responses were recorded to (−)-limonene, while (+)-limonene elicited weaker responses. An intermediate response was recorded to racemic limonene.

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VARIABLES AFFECTING PHEROMONE CONCENTRATION IN VINEYARDS TREATED FOR MATING DISRUPTION OF GRAPE VINE MOTH *Lobesia botrana*

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Abstract—Airborne pheromone concentration in a field is one of the most important variables for the successful application of mating disruption in pest control. In the present paper, we estimated the pheromone concentration with field EAG recordings in vineyards and investigated parameters affecting concentration. Pheromone concentration showed a positive correlation with number of dispensers per hectare (= number of point sources). A twofold increase in the absolute number of dispensers per hectare with a constant number of point sources (two dispensers at the same location) did not significantly affect relative pheromone concentration. Measurements carried out in plots where dispensers had been applied at different heights showed highest relative pheromone concentrations in plots with dispensers at 0.1 m and 1.4 m above the ground. Those concentrations were not significantly different from each other, but were significantly higher than in plots where dispensers had been placed at a height of 2 m. Foliage of grape vines substantially affected the development of high pheromone concentrations. In summer, in vineyards with fully developed leaf canopy, significantly higher pheromone concentrations could be measured shortly after application of the dispensers compared to vineyards in spring with sparse vegetation. The decline of pheromone concentrations after removal of dispensers is significantly prolonged in full vegetation, showing the impact of plant canopy on pheromone concentrations. In contrast, ground cover between the grapevine rows did not significantly affect mean pheromone concentrations.

Key Words—*Lobesia botrana*, pheromone, mating disruption, field-electroantennogram, EAG, vineyard, plant canopy.

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INTRODUCTION

In mating disruption of Lepidoptera, the behavior of a male seeking a female is disturbed by exposure to synthetic pheromone released by dispensers (e.g., Bartell, 1982; Jutsum and Gordon, 1989; Ridgway et al., 1990; Cardé, 1990). For successful application of mating disruption in pest control, the concentration of synthetic pheromone and structure of the pheromone plume are important in the area to be protected. In general it is assumed that higher airborne pheromone concentrations in the treated areas lead to improved control of the pest. Relative pheromone concentrations and the structure of pheromone plumes have been measured with field electroantennograms (field EAG) (Sauer et al., 1992) in vineyards (Karg and Sauer, 1992, 1995, 1997) and orchards (Karg et al., 1994; Suckling et al. 1996; Suckling and Angerilli, 1996; Suckling and Karg, 1996; Karg and Suckling, 1997a, b). These experiments demonstrated the impact of plant canopy on mean pheromone concentrations by uptake and release of pheromone (Suckling et al., 1994; Karg et al., 1994, Suckling and Karg, 1996) and by forming protected areas and wind barriers. Significantly higher mean relative pheromone concentrations in vineyards were recorded in summer compared to spring. While pheromone concentrations measured at different locations inside vineyards were not significantly different from each other, concentrations declined rapidly outside the vineyard boundaries and above the canopy. In orchards, EAGs recorded in tree rows indicated a higher pheromone concentration in the vicinity of foliage. Pheromone buffering capacity of apple leaves also reduced trap catch and had an impact on the success of the mating disruption method in apple orchards (Suckling et al., 1996).

In addition, spatial and temporal distribution of pheromone in treated plots strongly depended on the plant canopy (Karg and Sauer, 1992, 1997; Suckling et al., 1994; Karg and Suckling, 1997a, b). Rather uniform distribution of pheromone occurred inside vineyards when the plant canopy was fully developed (Karg and Sauer, 1992, 1995, 1997). In contrast, strong fluctuations in pheromone concentrations were recorded in vineyards with sparse vegetation, e.g., in spring (Karg and Sauer, 1992, 1997). A positive correlation between the number of dispensers and the frequency of pheromone fluctuations in orchards was shown by Suckling and Angerilli (1996) and Karg and Suckling (1997a, b). When dispensers were removed, fluctuations instantly disappeared; however, trap catches were still significantly lower than in untreated orchards. This indicated that mating disruption worked without the presence of fluctuations. These authors hypothesized that stable pheromone concentrations, although low, are more important for the success of mating disruption than the presence of multiple odor plumes.

In this paper we investigated variables that influence pheromone concentrations in treated vineyards. We follow the paradigm that higher pheromone

concentrations increase mating disruption. With the help of this investigation we hope to improve the selection of the location for pheromone dispensers by examining the influence of dispenser placement on mean pheromone concentration.

METHODS AND MATERIALS

Insects. Lobesia botrana (Lepidoptera: Tortricidae) were reared in the Landwirtschaftliche Versuchsanstalt of BASF AG, Limburgerhof, and supplied to us as pupae. They were kept at approximately 20°C until eclosion and then stored at 9°C in a refrigerator until used in experiments. Antennae of 2- to 4-day-old males were used for the EAG recordings.

Pheromone. Dispensers were the standard RAK2 dispensers supplied by BASF AG, Ludwigshafen, Germany, and contained 0.1 g of (*E*, *Z*)-7,9-dodecadien-1-yl acetate (*E*, *Z*-7,9-12:OAc) the main pheromone component of *L. botrana* (Buser et al., 1974).

Field Sites. All experiments were carried out in vineyards in Billigheim, Rhineland-Palatinate, Germany. Plot sizes varied between 300 m^2 and 1.2 ha. The canopy height of the vineyards was approximately 2.1 m and interrow distances were approximately 2.2 m. Dispensers were placed in the field at least one week before the recordings commenced. In a standard plot the dispensers were applied to a branch of a grape vine at a height of approximately 1.4 m, in every second row with an approximate separation of 5 m within one row. All measurements commenced in untreated control vineyards, 350 m upwind from the pheromone treated fields, where the antennal responses to environmental volatiles (e.g., from host plants) were measured. The EAG system was then transported to the physical center of the pheromone-treated fields and set up at a maximum distance from the surrounding dispensers for measurement of the airborne pheromone. Recordings were generally taken in the late afternoon and early evenings (4 and 10 PM) on several occasions between July 5 and August 30. All measurements were taken under comparable climatic conditions, including wind speeds of 0–1.5 m/s and temperatures of 19.5–24°C.

Field EAG Apparatus. The field EAG apparatus is described in full detail in Sauer et al. (1992), Karg et al. (1994), and Karg and Sauer (1995, 1997). Air containing pheromone is sucked over an antennae mounted in a Perspex holder inside a glass chamber. The change of electrical potential of the antenna (EAG) is normalized to a calibration stimulus by dividing field antennal responses by the EAG response to a calibration pulse of 10^{-4} pheromone in paraffin oil. This value indicates "relative pheromone concentration" of the ambient air (Sauer et al., 1992; Karg and Sauer, 1995, 1997). Comparison of the normalized EAG amplitude elicited by ambient air with a dose-response curve allowed the

calculation of the airborne pheromone concentration. In experiments with different dispenser heights (see below), the whole apparatus was mounted on a small table and the inlet of the field EAG apparatus was at 1.2 m above the ground.

Comparison of Pheromone Concentration in Plots with Different Numbers of Dispensers and Point Sources. Pheromone dispensers were applied in three replicate blocks at rates of 1600, 400, and 180 dispensers/ha. In this experiment, the number of dispensers and the number of point sources were altered simultaneously. Mean relative pheromone concentrations were measured in the center of these plots one week after application and compared.

Comparison of Pheromone Concentration in Plots with the Same Number of Point Sources but Different Numbers of Dispensers per Point Source. In these measurements the number of point sources was kept constant at approximately 400/ha. In three plots each point source consisted of a single dispenser, while in three other plots two dispensers were applied per point source. Relative pheromone concentrations were measured by field EAG and compared. Measurements ($N = 14$) were carried out one week after the dispenser had been applied.

Dispenser Height. The impact of the height of the dispensers was measured in plots in which the dispensers had been applied at 0.1, 1.4, and 2 m above the ground. In each location the mean relative pheromone concentration was recorded at least 12 times.

Influence of Plant Canopy Between Rows (Ground Cover) on Pheromone Concentration. The foliage of the grapevines adsorbs and releases pheromone. Ground cover vegetation in vineyards prevents soil erosion and might have a positive effect on the population of beneficial insects. On the other hand, there may be a negative effect of ground cover on grapevines in the form of competition for nutrients (H. Rothhass, personal communication). In this study we investigated the influence of the canopy of the ground cover (grass) on the pheromone concentration in treated vineyards. The height of the canopy was 20–30 cm. In two adjacent plots, 400 dispenser/ha were applied. In one plot grass was left as ground cover, while in the other plot the soil had been ploughed. Mean relative pheromone concentrations were recorded in the center of the plots and compared.

Development of Pheromone Concentration. The increase and decline of pheromone concentrations were recorded in spring (May 10–21, mean temperature during the measurements: 20.5°C) and in summer (August 11–23, mean temperature during the measurements: 23°C) in plots with sparsely developed foliage and in plots with fully developed foliage. In order to exclude the influence of climatic parameters on the results, control experiments were made in a plot defoliated by a hailstorm.

The EAG apparatus was placed near the physical center of untreated plots,

close to a row of grapevines. First, EAGs were recorded in the untreated plots ($N > 5$). Then 25 dispensers were applied equidistantly (2.5 m) while EAGs were measured. Approximately 3 mins was required for application of the dispensers. The recordings were terminated after 5–7 min. EAGs were recorded again at the same location after 30 min with a new antenna and uncontaminated glassware. Three series of five measurements were carried out while the dispensers were still present, and then the pheromone dispensers were again removed from the plots while the EAGs recordings continued. These experiments were carried out in spring on three different days in three different plots ($N = 3$), and repeated in summer in vineyards with fully developed foliage ($N = 3$). In order to compare the time course of the mean relative pheromone concentrations between runs, the recorded concentrations of each set of recordings were normalized to the maximum pheromone concentration measured in a given run.

Statistical Analysis. EAG values were normally distributed. The mean pheromone concentrations were compared using Student's t test (Sachs, 1992). The differences in the mean concentration with dispensers applied at different heights were tested with ANOVA and the Tukey test (Sachs, 1992). In all figures the vertical bars indicate the standard deviation of the mean.

RESULTS

Comparison of Pheromone Concentration in Plots with Different Numbers of Dispensers and Point Sources. The pheromone concentration depends on the number of pheromone dispensers per hectare (Figure 1). Relative pheromone concentrations were highest in plots with 1600 dispensers/ha and reached a mean 3.1×10^{-4}. In the plot in which dispensers had been applied at a density of 400 dispensers/ha, the mean concentration was 8.6×10^{-5}, but this was not significantly different from the 1600/ha plot ($P > 0.05$) due to the large variance between measurements. However, this value corresponded nicely with the expected fourfold lower mean concentrations that might be expected with only 400 dispensers/ha. At 180 dispensers/ha, a mean concentration of 4.2×10^{-6} was recorded, which was significantly lower compared to the concentrations measured in the plots containing 1600 or 400 dispensers/ha ($P < 0.001$). The expected reduction in mean concentration compared to the 400/ha block was 2.3-fold, but the results showed a 20-fold reduction. Calculation of the correlation coefficient revealed a slightly better fit for the log-linear relationship ($y = 0.0003 \ln(x) + 0.0003$, $R^2 = 0.98$) compared to a linear relationship ($y = -0.0002x + 0.0004$, $R^2 = 0.93$).

Comparison of Pheromone Concentration in Plots with the Same Number of Point Sources but Different Numbers of Dispensers Per Point Source. In this experiment we compared the pheromone concentrations in a plot with the stan-

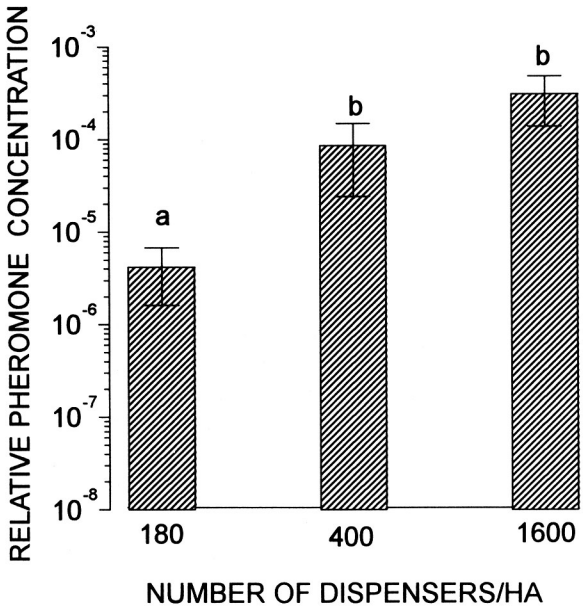

FIG. 1. Comparison of mean (±SD) relative pheromone concentrations in vineyards treated with 180, 400, and 1600 pheromone dispensers/ha. Histograms with different letters are significantly different from each other.

dard application (400 dispensers/ha = 400 point sources) and a plot with two dispensers per point source but the same distance between the point sources (800 dispensers/ha, with two at each of 400 point sources) as in the previous plot. The mean relative pheromone concentration in the standard plot was 8.5 $\times 10^{-5}$ compared to 1.8 $\times 10^{-4}$ in the plot with the double dispensers. The twofold increase in the number of pheromone dispensers at a constant number of point sources did not result in a significant increase in the pheromone concentration ($P > 0.05$; Figure 2).

Dispenser Height. Application of dispensers at different heights above the ground (0.1, 1.4, and 2 m) showed that concentrations were highest in the plots where the dispensers had been applied at 0.1 m and 1.4 m (Figure 3). Concentrations at 0.1 m and 1.4 m were 2.3 $\times 10^{-5}$ and 1.2 $\times 10^{-5}$, respectively, and were not significantly different from each other ($P > 0.05$). Application at a height of 2 m resulted in a significantly lower mean pheromone concentration of 1.0 $\times 10^{-6}$ ($P < 0.001$) when compared to the concentrations at the 0.1-m and 1.4-m heights of the dispensers. Analyses were done by one-way ANOVA ($F_{3,44} = 5.72$, $P > 0.05$) followed by the Tukey test.

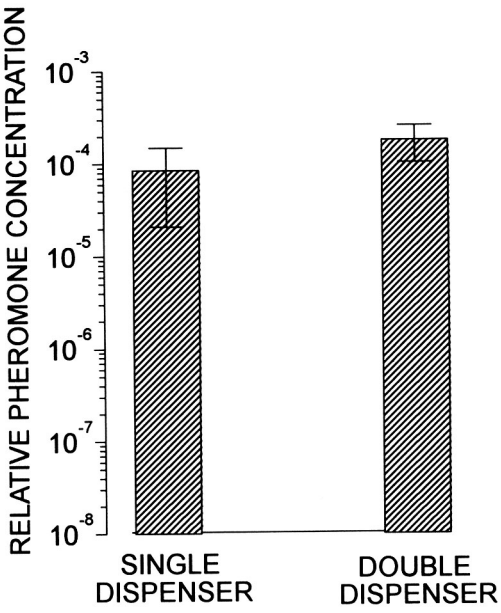

FIG. 2. Effect of doubling the number of dispensers at each location on the mean (±SD) relative pheromone concentrations in a vineyard treated with 400 dispenser/ha at 400 pheromone point sources and a vineyard treated with 800 dispensers/ha at 400 point sources. Mean pheromone concentrations do not differ significantly ($P > 0.05$).

Influence of Ground Cover (Plant Canopy Between Rows) on Pheromone Concentration. Ground cover/understory did not significantly affect the mean pheromone concentration in spring in vineyards with sparse vegetation ($P > 0.05$) or in summer in fully developed vineyards ($P > 0.05$) (Figure 4). Mean relative pheromone concentration in spring was 8.8×10^{-7} in vineyards without ground cover and 6.8×10^{-7} in nearby vineyard with grass ground cover. In summer, the mean pheromone concentration in vineyards without ground cover was 1.8×10^{-4} and 2.3×10^{-4} in vineyards with grass ground cover.

Development of Pheromone Concentration. Application of dispensers in the vineyards led to an immediate increase in the mean pheromone concentration within minutes (Figure 5A) and removal of dispensers led to a rapid decline (Figure 5B). Comparison of the time course of the mean relative pheromone concentrations (normalized to the maximum pheromone concentration recorded in each set of measurements) showed a significant difference ($P < 0.001$) (see below) in the time course of the decline of the pheromone concentration between spring, when the canopy is only sparsely developed (stages 07–12; Eichhorn

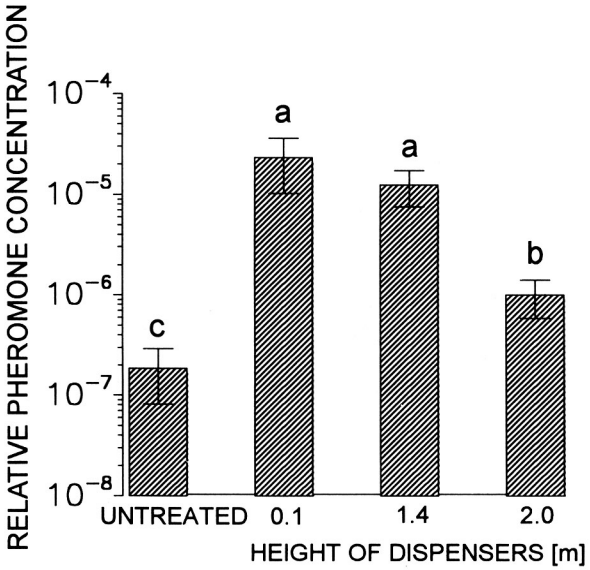

FIG. 3. Comparison of mean (±SD) relative pheromone concentrations in untreated vineyards with vineyards treated with 400 dispensers/ha applied at different heights above the ground. Mean relative pheromone concentrations were measured at 1.2 m height. The pheromone concentrations from dispenser heights of 0.1 and 1.4 m were not significantly different from each other ($P > 0.05$). With dispensers at a height of 2.0 m, a significantly lower pheromone concentration was measured ($P < 0.001$). Histograms with different letters are significantly different from each other.

and Lorenz, 1977) compared to summer, with fully developed foliage (stages 27–35; Eichhorn and Lorenz, 1977).

In spring, approximately 2 min after application of the dispensers, the mean relative pheromone concentration reached a plateau (between 1.4×10^{-6} and 4.4×10^{-6}, values not shown in the figure) and did not change significantly during the following 30 min ($P < 0.001$). Pheromone concentrations that were not significantly different ($P > 0.05$) were also measured in vineyards in which the dispensers had been left for three days. Removal of the dispensers after they had been applied in the vineyard for only 30 min led to a rapid decline in the mean pheromone concentration (Figure 5, $N = 3$). Only 2–5 min after the removal, EAG responses were significantly ($P < 0.05$) reduced and reached values that were not significantly different from the ones recorded in untreated control blocks.

In summer the mean relative pheromone concentration rose to significantly higher concentrations (between 4.4×10^{-5} and 1.8×10^{-4}) compared to the

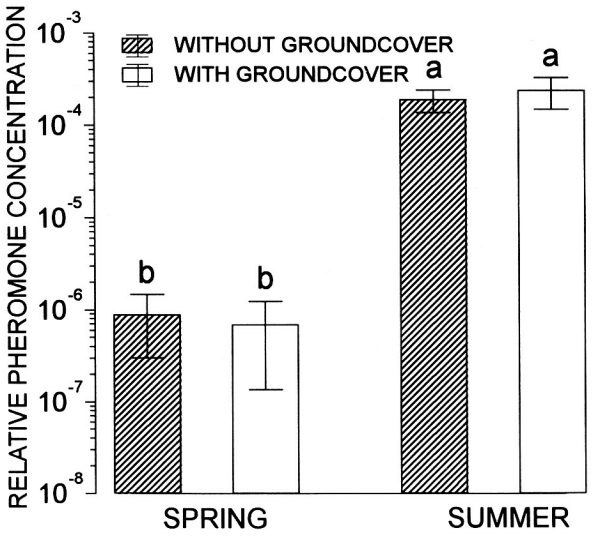

FIG. 4. Effect of ground cover on mean (±SD) relative pheromone concentrations in spring and summer. Pheromone concentrations are not significantly different ($P > 0.05$).

values in spring in approximately 7–8 min ($P < 0.001$). However, the slope of a regression line fit for the first five values was only slightly higher in vineyards with reduced plant canopy (0.30/100 sec) compared to vineyards with intact canopy (0.23/100 sec) ($y_{summer} = 0.0023x + 0.46$ compared to $y_{winter} = 0.0030x + 0.55$). Removal of the dispensers led to a rapid decline in the mean pheromone concentrations in summer and spring. However, the decline was significantly slower in summer compared to spring ($P < 0.001$). EAG responses measured 2 hr after removal of the dispensers in the summer blocks were still significantly higher compared to untreated control blocks ($P < 0.001$).

The situation in the defoliated plot reflects the spring situation. Pheromone concentrations between 1.9×10^{-6} and 4.6×10^{-6} were reached after 2–5 min, and the concentrations did not rise any further.

DISCUSSION

There are a number of factors that may hamper the success of mating disruption that cannot be discussed here in detail. However, one of the assumptions generally agreed on is that insect mating is more effectively disrupted with increasing airborne pheromone concentration. In this paper we have determined additional variables that influence pheromone concentrations that will have to

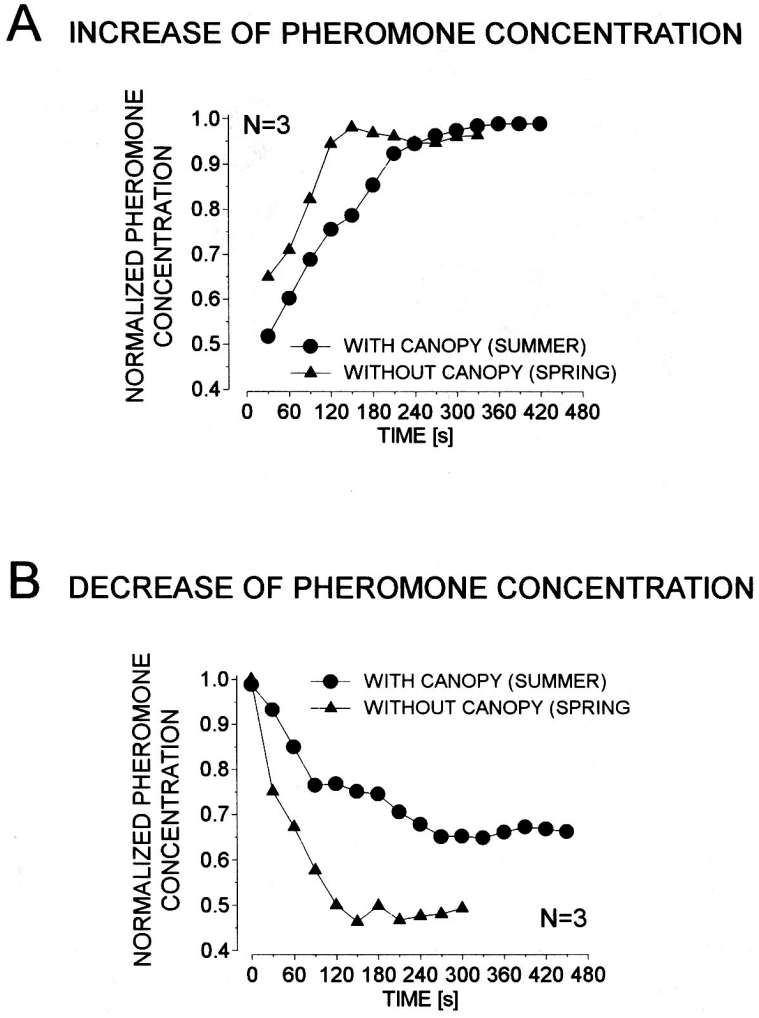

FIG. 5. Development and decay of pheromone concentration after application (A) and removal (B) of pheromone dispensers. The curves show the time course of the mean relative pheromone concentrations (normalized to the maximum pheromone concentration recorded in each set of measurements) in vineyards with fully developed canopy (summer, circles) and in vineyards without canopy (spring, triangles). Values are the means of three measurements. The decay of mean relative pheromone concentration is significantly prolonged in vineyards with fully developed grapevine canopy ($P < 0.001$).

be taken into account and that can be modified easily in order to maximize pheromone concentrations in plots.

Pheromone concentrations measured in plots containing 1600 or 400 dispensers/ha (distance between dispensers 2.5 and 5 m, respectively) did not vary significantly, but an unproportional drop in the mean concentrations occurred when only 160 dispensers/ha (distance between dispensers 7.5 m) were applied. From these results we conclude that the active space of a single dispenser, i.e., the distance between dispensers that is required for stable pheromone concentrations), has a radius of approximately 5 m. Application of limited numbers of dispensers over a greater distance (e.g., 180 dispensers/ha) will lead to a significant and nonproportional drop in pheromone concentration. While the active spaces of dispensers do not overlap at distances greater than 5 m (radius), this is the case at higher densities. The overlap of active spaces between dispensers will lead to an even distribution of pheromone in the entire plot. These data corroborate results obtained by Karg et al. (1994) in orchards. In experiments where 185 point sources had been applied per hectare, they measured a drop in the mean relative pheromone concentrations between dispensers. In practice, missing grape vines or trees could be a potential problem because they disturb the distribution pattern of the dispensers and might allow pheromone concentrations to drop below the disruption level required.

According to our results an even distribution within a vineyard can best be achieved when the BASF dispensers are applied in standard mating disruption trials approximately 5 m apart. Fortunately, this is the standard application rate that has empirically been established during the development of mating disruption of *L. botrana* and *Eupoecilia ambiguella* in German vineyards.

However, Charmillot et al. (1995a, b) successfully obtained mating disruption of *L. botrana* in plots that did not have 5-m dispenser spacing. In their experiments the plots had high numbers of dispensers along the edges of the plots (1-m distance between dispensers) but reduced numbers in the center. In that study the population within the plots had been reduced over several years and inflow of insects from the surrounding area was prevented by the application of a high number of dispensers at the edge of the vineyard. We assume that in their case the insects could be disrupted successfully with lower pheromone concentrations inside the plots because population densities were substantially reduced in the plot by high concentrations along the edge of the plots.

In our experiments a twofold increase in the number of pheromone dispensers at a constant number of point sources did not result in a significant increase in the pheromone concentration. One explanation for this result could be the variance in the individual measurements. The resolution of the EAG is limited and the twofold difference in the mean relative pheromone concentrations cannot be detected easily.

The airborne pheromone concentration in a plot depends, among other variables, on the application height of the dispensers. Generally speaking, pheromone concentrations will be highest at the dispenser height, which was shown by using chemical analysis (Caro et al., 1980) as well as electroantennogram recordings (Karg and Sauer, 1995). The difference in pheromone concentrations in plots with different dispenser heights is related also to changes in wind speed, which generally increases with height above ground (Raupach, 1988; Green et al., 1995). Higher wind speeds produce higher wind shear and greater vertical mixing, which leads to a greater loss of pheromone and smaller mean relative pheromone concentrations. Caro et al. (1980) found that with decreasing wind speed, pheromone concentration increased sharply.

To achieve the highest pheromone concentrations, we propose that dispensers should be placed well within the plant canopy to reduce pheromone loss through wind shear and enhance uptake of pheromone by foliage. However, other variables such as the leaf area index will also have to be taken into account. Other factors will also play a major role in the practical application of the dispensers in vineyards. In spring, after the grape vines have been pruned, usually only one or two shoots will be left on the vines and thus available for attachment of pheromone dispensers.

The uptake and release of pheromone by foliage has been shown in a number of publications (Wall et al., 1981; Karg et al., 1994; Suckling et al., 1996). They found that the most likely sites for uptake and release of pheromones by plants are the waxy surfaces of the leaves (Karg et al., 1994). The ground cover in vineyards, which consists mainly of grass, has only a very thin layer of wax (if any) and the surface area is rather small. We cannot exclude that ground cover in the vineyard took up pheromone, but the uptake and release is probably minimal and below the detection level of our apparatus. Ground cover from plants with thicker layers of wax might have a greater impact on pheromone concentrations.

The mean relative pheromone concentrations measured in summer were significantly higher compared to those measured in spring. Our results also showed that the mean EAG signal declined linearly after removal of dispensers from treated vineyards. Our data corroborate earlier experiments in orchards, which indicated a log-linear rate of drop in mean pheromone concentration (Karg and Suckling, 1997). Measurements taken in spring, when there is little foliage present, compared to measurements taken in summer, during fully developed foliage, show that the decline of mean concentration in summer requires a significantly longer time. This can be explained by the effect of foliage that functions as a wind barrier as well as a secondary pheromone dispenser.

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SPECIFICITY OF SYSTEMICALLY RELEASED COTTON VOLATILES AS ATTRACTANTS FOR SPECIALIST AND GENERALIST PARASITIC WASPS

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Abstract—Cotton plants under herbivore attack release volatile semiochemicals that attract natural enemies of the herbivores to the damaged plant. The volatiles released in response to herbivory are not only released from the damaged leaves but from the entire cotton plant. We found that cotton plants that released myrcene, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, and (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene systemically from undamaged leaves of caterpillar damaged plants were attractive to the generalist parasitoid *Cotesia marginiventris* and the specialist parasitoid *Microplitis croceipes*. Plants from which the caterpillar damaged leaves were removed and that released those compounds systemically were significantly preferred over undamaged control plants in two-choice experiments in a flight tunnel. Artificially damaged cotton plants that released green leafy volatiles and constitutive terpenoids were less attractive for *M. croceipes* and *C. marginiventris*. Only *C. marginiventris* preferred artificially damaged plants over undamaged control plants, whereas *M. croceipes* showed no preference. The apparent lack of specificity of systemically released compounds in response to different herbivores feeding on the lower leaves is discussed.

Key Words—*Gossypium hirsutum*, cotton, parasitoids, *Microplitis croceipes*, *Cotesia marginiventris*, plant-insect interactions, volatile semiochemicals, systemic induction, plant defense, host-searching behavior, generalist, specialist.

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INTRODUCTION

Several plant species are known to release volatile compounds when under herbivore attack (Dicke et al., 1990a; Turlings et al., 1990; McCall et al., 1994; Loughrin et al., 1994). This herbivore-induced release of volatiles benefits the plant by attracting natural enemies of the herbivores that feed on its foliage and benefits parasitoids and predators by guiding them to potential hosts or prey on the plant (Dicke and Sabelis, 1988; Dicke et al., 1990b; Turlings et al., 1991a,b; Takabayashi et al., 1991; McCall et al., 1993). The volatile compounds released from damaged plants can be divided into constitutive compounds and inducible compounds. Constitutive compounds are continuously present in the plant and are released from damaged leaves immediately after the beginning of feeding damage or even after the plant is only artificially damaged with a razor blade to mimic the mechanical part of feeding damage (Turlings et al., 1990; Röse et al., 1996). Early stages of plant damage are characterized by the release of "green leafy" volatiles such as (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, and the constitutive compounds that are plant specific (McCall et al., 1994; Loughrin et al., 1994; Turlings et al., 1995). Constitutive compounds in cotton are monoterpenes and sesquiterpenes (Loughrin et al., 1994) that are stored in lysigenous glands (Elzen et al., 1985). After several hours of herbivore damage or on the next day, the plants start to release additional compounds that appear to be specifically released in response to herbivore damage. Their release from damaged leaves is not elicited in significant amounts by artificial damage alone (Turlings et al. 1990; Paré and Tumlinson, 1997a; Röse et al., unpublished data). These herbivore-inducible compounds in cotton are acyclic terpenoids [i.e., (*E*)-β-ocimene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene] and (*Z*)-3-hexenyl acetate, indole, isomeric hexenyl butyrates, and 2-methylbutyrates (McCall et al., 1994; Loughrin et al., 1994).

Several of the inducible compounds in cotton are not only released at the damaged site, but are released systemically throughout the entire plant (Röse et al., 1996). After feeding damage of beet armyworm larvae *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) on the lower leaves of a cotton plant for several days, the upper undamaged leaves of the same plant released (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Röse et al., 1996).

In cotton, the release of inducible terpenes [(*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene] from the entire damaged plant follows a diurnal cycle, with peak emission of volatiles in the early afternoon (Loughrin et al., 1994). The timing of the plant signal coincides

with the time of active foraging of parasitic wasps (Snow and Burton, 1967; Lewis et al., 1972; Turlings et al., 1995), and several parasitoids and predators are known to exploit herbivore-induced compounds to lead them to those plants where they are likely to encounter a host or prey (Dicke and Sabelis, 1988; Turlings et al., 1990; Dicke and Dijkman, 1992; McCall et al., 1993).

The systemic release of inducible compounds in cotton plants allowed us to separate the attraction of parasitoids to inducible compounds from the attraction to constitutive compounds in behavioral bioassays in the flight tunnel. We conducted experiments with the generalist parasitoid *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) and the specialist parasitoid *Microplitis croceipes* Cresson (Hymenoptera: Braconidae) to observe whether the systemic release of inducible compounds would increase the attractiveness of the cotton plant for the parasitoids. *C. marginiventris* can parasitize larvae of the noctuid species *S. exigua*, *Spodoptera frugiperda* J. E. Smith, and *Helicoverpa* and *Heliothis* spp., whereas *M. croceipes* is an endoparasitoid of *Heliothis* and *Helicoverpa* spp. but cannot develop in *S. exigua* larvae (Blumberg and Ferkovich, 1994). *S. exigua* larvae were used to induce the systemic volatile release for our flight-tunnel experiments. To confirm that cotton plants that were used for flight-tunnel experiments released inducible compounds, their volatiles were collected immediately prior to the flight-tunnel experiments. In addition, we determined the specificity of the systemic plant signal to the attacking herbivore species. In a second set of two-choice flight tunnel experiments, we compared the attractiveness to the parasitoids of artificially damaged cotton plants that released constitutive volatile compounds and undamaged control plants.

METHODS AND MATERIALS

Plants. Approximately 6-week-old cotton plants, *Gossypium hirsutum* L. (Malvaceae), var. Deltapine acala 90, with six fully developed leaves in addition to the two cotyledons, were used in all experiments. Cotton was grown in a greenhouse in a mixture of compost, peat moss, and vermiculite (Metro-mix 300, Scotts-Sierra Horticultural Company, Marysville, Ohio) with natural light, under Florida summer conditions (14L:10D light cycle, 85 $\pm$ 10% relative humidity, and 35 $\pm$ 10°C). Each plant was grown from seed planted in a 16-cm-diameter pot and fertilized once at time of planting with a three to four-month formulation of Osmocote 14-14-14 (N-P-K) controlled-release fertilizer (Scotts-Sierra Horticultural Products).

Lepidoptera Larvae. Beet armyworm larvae (BAW) and corn earworm (CEW) *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) larvae were obtained from the USDA rearing facilities in Gainesville, Florida. Larvae were reared

on an artificial diet based on pinto beans according to the method of King and Leppla (1984). To encourage immediate feeding of larvae after being caged on leaves, third instars were starved for 24 hr prior to the experiments.

Parasitoids. The specialist larval endoparasitoid *M. croceipes* and the generalist larval endoparasitoid *C. marginiventris* were reared from cocoons obtained from colonies maintained at the US Department of Agriculture-Agricultural Research Service, Insect Biology and Population Management Research Laboratory, Tifton, Georgia. *M. croceipes* were reared on larvae of *H. zea* fed on CSM diet (Blended Food Product, Child Food Supplement, Formula No. 2) as previously described (Burton, 1970; Lewis and Burton, 1970). *C. marginiventris* of the 1985 Mississippi strain were reared on larvae of *S. frugiperda* fed on soybean diet as described by Lewis and Burton (1970). Female and male parasitoids were kept together in screen cages (25 × 25 × 25 cm) in the laboratory to allow mating and were fed with honey and water after emergence. Females were separated in cages according to their date of emergence. Mated females used for flight tunnel experiments were 3–4 days old and kept in the laboratory at 24 ± 2°C, 55 ± 5% relative humidity, and on a 14L:10D light cycle.

Caterpillar Feeding on Lower Cotton Leaves. To determine the specificity of the systemically released chemicals after herbivore attack, volatiles were collected from the undamaged upper leaves after feeding damage of CEW on the lower leaves and compared to compounds systemically released after feeding damage of BAW larvae. For this, a total of either four BAW or four CEW were placed on the two lower leaves of separate plants. On each leaf, two BAW or two CEW were enclosed in a cage that consisted of two modified halves of a Petri dish as previously described (Röse et al., 1996). BAW or CEW larvae were placed on the lower leaves on day 1 at 11:00 hr and were replaced with new, starved larvae every day for four days. On day 4 at 11:00 hr, volatiles were collected from the undamaged upper leaves of the BAW-damaged plant (SYST-BAW) and from the undamaged upper leaves of the CEW-damaged plant (SYST-CEW) for 1 hr as previously described (Röse et al., 1996). Volatiles were also collected from upper leaves of undamaged control plants (SYST-CTRL) in the same way.

Artificially Damaged Cotton Plants. For the collection of volatiles from artificially damaged cotton plants (ART), the third and fourth leaves (counted starting from the youngest leaves) were damaged in the center with a garlic press (Ekco Housewares, Franklin Park, Illinois), inflicting 32 holes of approximately 1 mm^2 each over an area of 15 × 35 mm/leaf. Volatile collections from ART plants that included the damaged leaves were conducted from 12:00 to 15:00 hr on different plants from the ones used for flight-tunnel experiments. Separate plants were used to avoid a decrease in volatiles passively released from the damaged leaf. Volatiles were also collected from undamaged control plants (CTRL) in the same way from 11:00 to 15:00 hr.

Volatile Collection. To collect volatiles from SYST-BAW, SYST-CEW, and SYST-CTRL, the upper four leaves of each plant were enclosed in separate volatile collection chambers as previously described (Röse et al., 1996). These were part of an automated volatile collection system as previously described (Heath and Manukian, 1992, 1994; Manukian and Heath, 1993). This system allows for the collection of volatiles from the upper portion of the plants while completely isolating the lower section of the plant where caterpillars are feeding in the systemic treatment.

Volatiles emitted from the upper portion of the plant enclosed within the glass chamber were swept downward by the incoming pure laminar airflow at a rate of 5 liters/min. They were sampled at the bottom of the chamber by pulling air at a rate of 1 liter/min through volatile collection traps (Super-Q, 50 mg) (Röse et al., 1996) with a controlled vacuum source attached to each volatile collector trap from the automated volatile collection system. Thus, 20% of the air passed through the collector traps during the 1-hr collection period. The remaining 80% excess air escaped through a small opening around the stem of the plant at the bottom of the collection system. This positive pressure venting provided a barrier against all ambient air and prevented volatiles from the lower, damaged part of the plant from entering into the collection chamber containing the upper, undamaged part of the plant. Details of the release of volatiles from the undamaged parts of a caterpillar damaged plant and a control plant over four days were reported by Röse et al. (1996).

Volatiles from ART plants were collected with the same collection system from 12:00 to 15:00 hr, by enclosing all cotton leaves in the collection chamber, including the artificially damaged ones, while the pot remained outside the system. These plants were artificially damaged on the third and fourth leaf and immediately placed in the chamber for collection of 20% of the volatiles. Volatiles were collected at the same time from CTRL plants.

Analysis of Volatiles. Volatiles were extracted from the collector traps by washing with 170 μl methylene chloride (capillary GC/GC-MS solvent, Burdick & Jackson, Muskegon, Michigan). Internal standards (600 ng each of *n*-octane and nonyl acetate in 60 μl methylene chloride) were added to the extract. Of each collection sample, 1 μl was analyzed on a Hewlett-Packard gas chromatograph (model 5890 II plus). Samples were injected by a Hewlett-Packard auto injector (model 6890) in split-splitless mode. The GC was equipped with a flame ionization detector. Data collection, storage, and subsequent analysis was performed on a Perkin Elmer chromatographic data system. Helium at a linear flow velocity of 20 cm/sec was used as a carrier gas. All samples were analyzed on a fused silica capillary column (Quadrex Corporation, New Haven, Connecticut), 50 m $\times$ 0.25 mm ID, with a 0.25-μm-thick film of bonded methyl silicone (007). The temperature of the column oven was maintained at 40°C for 3 min, and then programmed at 5°C/min to 220°C, which was maintained for

10 min. The injector temperature was set at 220°C, the detector temperature at 260°C.

To identify compounds, volatiles were analyzed by GC–mass spectroscopy (GC-MS) with a Finnigan ITS-40 Magnum (ion-trap) mass spectrometer operated in electron impact and chemical ionization modes. For GC-MS, a fused silica capillary column was used with helium as a carrier gas, and for chemical ionization, isobutane was used as reagent gas. Constituents of the plant volatile emission were identified by comparison of mass spectra with spectra in the Environmental Protection Agency–National Institutes of Health data base, the Environmental Protection Agency–National Institute of Standards and Technology data base, and spectra obtained from authentic compounds. GC retention times of plant volatiles also were compared with GC retention times of authentic compounds on the methyl silicone column.

Flight Tunnel. All free flight experiments with *M. croceipes* and *C. marginiventris* were carried out in a Plexiglas flight tunnel 60 × 60 cm in cross section and 240 cm long, as previously described (Eller et al., 1988; Turlings et al., 1991a). The windspeed was adjusted to 0.2 m/sec. Air was exhausted outside the building after being drawn through the tunnel. A pattern of 20-cm-wide black and white stripes under the tunnel was used to provide flying insects with a visual reference. Four 90-W krypton lights illuminated the flight tunnel with approximately 800 lux from above (measured with an Extech Instruments light meter, Davis Instruments, Baltimore, Maryland). A temperature of 27 ± 1°C, and 75 ± 5% relative humidity was maintained in the tunnel during the experiments. Wasps used for flight-tunnel experiments were transferred to the flight tunnel room 3 hr prior to the experiment to adjust to the room conditions and were used 4–6 hr into the photophase. All wasps were used only once in an experiment.

Preflight Experience. To increase the responsiveness of the parasitoids, females of *M. croceipes* were first allowed to antennate and parasitize a third instar CEW fed on pinto bean diet and were immediately afterwards (within 3 min) released in the flight tunnel. Females of *C. marginiventris* were first allowed to antennate and parasitize a third-instar BAW fed on pinto bean diet. Wasps that were injured or came in contact with larval oral secretion during oviposition were discarded.

Flight-Tunnel Experiments. Only cotton plants that were fed upon by BAW and for which a systemic volatile release was confirmed by collection and analysis of volatiles were used for flight tunnel experiments (SYST-BAW). After the volatile collection, at 12:00 hr, SYST-BAW plants were prepared for the flight tunnel by removing caterpillars from the lower leaves and cutting the damaged leaves off to remove any volatile or visual cues resulting from the feeding site itself. The cut leaf stems of the plant were wrapped in Teflon tape to minimize the release of volatiles from the cut. SYST-CTRL plants were

treated in the same way, and two lower leaves that were matched for size and position with the SYST-BAW plants were removed. The cut leaf stems of the plant were wrapped in Teflon tape like SYST-BAW. Both the SYST-BAW and SYST-CTRL plant, with the lower leaves removed, were placed in the flight tunnel for two-choice flight experiments.

To compare ART plants and CTRL plants, initially only the third leaf (counted starting from the youngest leaves) of ART plants was damaged prior to placing the plant in the flight tunnel. After 1 hr of experiments, or after 12 wasps were flown in the flight tunnel, an additional leaf at the fourth position was damaged to ensure continuous release of volatiles. An undamaged cotton plant matched for size and leaf number was used as a control plant for two-choice experiments in the flight tunnel (CTRL).

No-Choice Flight Tunnel Experiments. To establish a baseline response of *C. marginiventris* and *M. croceipes* to undamaged cotton plants, we observed the number of completed flights to CTRL plants in a no-choice situation.

Two-Choice Flight Tunnel Experiments. All two-choice experiments compared the flight response to a treated plant and an undamaged control plant. The first set of experiments investigated the attraction of *C. marginiventris* and *M. croceipes* to herbivore inducible volatiles systemically released from SYST-BAW plants compared to SYST-CTRL plants. The second set compared responses to ART plants with responses to CTRL plants. These experiments were conducted to distinguish the responses of parasitoids to constitutive cotton volatiles from responses to inducible volatiles specifically released in response to herbivore damage. The position of the plants in the flight tunnel was switched routinely for each pair to avoid positional bias. Both plants were positioned equidistant from the parasitoid release point. Parasitoids were released individually 80 cm downwind of the odor source in a glass cylinder 25 cm above the tunnel floor. The glass cylinder ended in a curved funnel opening into a glass tube (details described by Turlings et al., 1991b) that was oriented parallel to the airflow. The odors released upwind could pass through the glass tube, but the tube prevented the insects from taking flight before experiencing the odors. In all bioassays, each parasitoid was given three chances to complete a flight by landing on a plant after a nonstop flight. After an incomplete flight, the parasitoid was returned to the release chamber to be released again. The choice of the parasitoid after a completed flight was recorded, as was the number of wasps that did not complete flights.

Statistical Analyses. The flight response of both parasitoid species was investigated on five separate days, with a total of 60 wasps tested for their attraction to the plants in each no-choice experiment (CTRL) and in each two-choice experiment (SYST-BAW vs. SYST-CTRL and ART vs. CTRL). Differences in the total numbers of wasps that completed a flight to a plant over all experiments and differences within each two-choice experiment in the total

number of wasps responding to each choice were analyzed by Fischer's exact test with the statistic program Systat (Systat Inc., Evanston, Illinois). The *t* test was used to determine differences in the amounts of volatiles released per plant from SYST-BAW and SYST-CEW leaves, for pairwise comparisons of those plants to ART and CTRL leaves, and for pairwise comparisons of SYST-CTRL and CTRL plants. Observed volatile amounts were summarized by the mean and corresponding standard error. Comparisons yielding $P \leq 0.05$ were considered to be statistically significant.

RESULTS

The total amount of volatiles released from the plants varied with the different treatments. While the totals released from both systemic treatments were similar (SYST-BAW: 12.58 μg/hr, SD = 6.8 μg/hr; SYST-CEW: 12.02 μg/hr, SD = 7.1 μg/hr) the totals released from ART (4.57 μg/hr, SD = 1.7 μg/hr) and CTRL (0.29 μg/hr, SD = 0.2 μg/hr) plants were both significantly ($P \leq 0.05$) smaller. Furthermore, the composition of the volatile blends released by the systemic treatments varied from the volatile composition released by ART and CTRL plants (Figure 1). Volatile compounds systemically released from SYST-BAW and SYST-CEW plants were mostly acyclic terpenoids [i.e., (*E*)-β-ocimene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene] and (*Z*)-3-hexenyl acetate. All these compounds were released in higher amounts from SYST-BAW and SYST-CEW plants compared to CTRL plants. In addition, small amounts of other inducible compounds [indole, (*Z*)-3-hexenyl butyrate] and small amounts of constitutive compounds were released.

Volatile compounds released from SYST-BAW and SYST-CEW showed no qualitatively and quantitatively significant differences (Figure 1A and B). Artifically damaged cotton leaves released green leafy volatiles [(*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate] and other constitutive compounds (α-pinene, β-pinene, myrcene, β-caryophyllene) (Figure 1C), whereas undamaged CTRL plants released only trace amounts of constitutive compounds and no measurable amounts of inducible compounds (Figure 1D). No differences were detected in the amounts of volatiles released from SYST-CTRL and CTRL leaves.

In the flight tunnel, both parasitoid species completed only 15–20% of flights to undamaged CTRL plants in a no-choice situation (Figure 2A and B; CTRL). These CTRL plants released only trace amounts of constitutive volatile compounds (Figure 1D). The total number of flights completed by the generalist *C. marginiventris* was significantly increased ($P \leq 0.032$) when an ART plant that released green leafy volatiles and constitutive compounds in larger amounts

(Figure 1C) was added to the flight tunnel for a two-choice experiment (Figure 2A; ART + CTRL). However, the addition of an ART plant to the CTRL plant did not increase the total number of flights completed by the specialist *M. croceipes* (Figure 2B; ART + CTRL). Parasitoids that were allowed to fly to SYST and SYST-CTRL plants showed a significant increase in the number of completed flights for *C. marginiventris* (Figure 2A; SYST + SYST − CTRL; $P \leq 0.01$) and *M. croceipes* (Figure 2B; SYST + SYST − CTRL; $P \leq 0.001$) when compared to ART and CTRL plants. SYST plants released high amounts of acyclic terpenoids (Figure 1A).

Besides differences between the parasitoid species in the total number of completed flights to the different plant treatments, we observed further differences in the choices the wasp species made between treated and control plants. The preference for SYST-BAW plants that released inducible volatile compounds over SYST-CTRL plants was highly significant for the generalist *C. marginiventris* (Figure 3A, $P \leq 0.001$) and the specialist *M. croceipes* (Figure 3C, $P \leq 0.001$). However, a difference between the two parasitoid species was observed when they were given a choice between ART and CTRL plants. The generalist *C. marginiventris* preferred ART plants that released constitutive volatile compounds over CTRL plants (Figure 3B; $P \leq 0.041$), whereas the specialist *M. croceipes* showed no preference for either plant (Figure 3D, $P \leq 1.0$).

DISCUSSION

The generalist parasitoid *C. marginiventris* and the specialist parasitoid *M. croceipes* both completed a small number of flights to undamaged cotton plants. Those undamaged plants released only trace amounts of volatiles and may be attractive to parasitoids by their color and shape alone. Wäckers and Lewis (1994) showed that *M. croceipes* uses visual as well as olfactory cues to locate a host site. The attraction to undamaged plants was displayed by the wasps after a preflight experience consisting of antennation and oviposition of a diet-fed host larva. This experience sensitizes and activates the host-searching behavior of a wasp and increases the number of completed flights compared to naive wasps (Röse et al., 1997). However, it does not provide the wasp with any cotton plant-related volatiles, nor does the parasitoid gain any experience with color or shape of a cotton plant. Therefore, the attraction of parasitoids to cotton plants is considered to be innate.

The attraction of the generalist *C. marginiventris* was greater to artificially damaged cotton plants than to undamaged plants. Artificially damaged and recently caterpillar-damaged cotton plants release green leafy volatiles and a

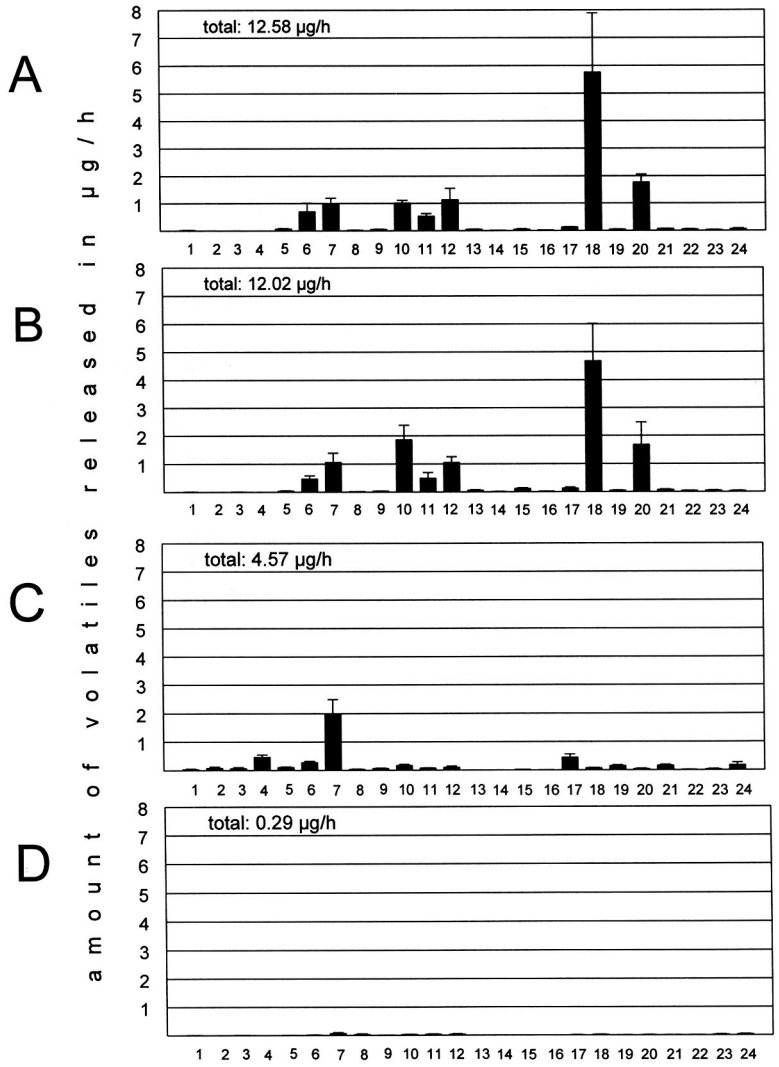

FIG. 1. Composition of volatile blends (mean over five replications with SEM) (A) systemically from undamaged leaves of a beet armyworm-damaged cotton plant after four days of feeding damage on the lower leaves (SYST-BAW), (B) systemically from undamaged leaves of a corn earworm-damaged cotton plant after four days of feeding damage on the lower leaves (SYST-CEW), (C) from artificially damaged cotton leaves (ART), and (D) an undamaged control plant (CTRL). Volatiles were collected from 11:00 to 12:00 hr for SYST-BAW and SYST-CEW, and from 12:00 to 15:00 hr for ART and CTRL plants. Volatiles were analyzed on a methyl silicone capillary column

number of constitutive mono- and sesquiterpenes that are different from the acyclic terpenoids that are released after several hours of herbivore damage (Loughrin et al., 1994; McCall et al., 1994). While the generalist *C. marginiventris* was increasingly attracted to plants that released those constitutive compounds, we did not observe an increase in the total number of completed flights for the specialist *M. croceipes* compared to the number of completed flights to control plants. A generalist like *C. marginiventris* can parasitize several different lepidoptera species that attack the same plant. Consequently, green leafy volatiles and constitutive compounds released from recently damaged plants may provide a useful cue for the location of a leaf-feeding host by the generalist. The specialist *M. croceipes* attacks only a limited number of lepidoptera larvae, one of them CEW, which prefers feeding on cotton squares and bolls. CEW-damaged cotton squares (Röse and Tumlinson, unpublished data) and bolls (Turlings et al., 1993a) release only small amounts of green leafy volatiles. Therefore, the chances of *M. croceipes* encountering a host on a plant that releases only green leafy volatiles and constitutive compounds are relatively small. This may explain the overall lower attraction to those compounds released from artificially damaged plants in our experiment.

Compared to the total number of flights completed by *M. croceipes* and *C. marginiventris* to artificially damaged plants, the level of response of the wasps to inducible volatiles systemically released in response to herbivore feeding was higher. *M. croceipes* and *C. marginiventris* responded strongly to cotton plants that released large amounts of inducible compounds systemically and preferred those plants over undamaged control plants. Because the wasps were again only allowed to antennate and sting a diet-fed host larva and did not have any previous experience with cotton volatiles, the attraction of the generalist and the specialist parasitoid species to systemically released cotton volatiles and the clear preference for those plants when compared to undamaged control plants appears to be innate. The innate preference of naive parasitoids of both species for plants that released inducible volatile compounds is high, whereas artificially or recently damaged plants that released constitutive compounds were less attractive to the wasps (Figure 2). Since inducible compounds are released in significant amounts

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and the amount of volatiles adjusted to the amount released per hour. Compound names: 1, (*Z*)-3-hexenal; 2, (*E*)-2-hexenal; 3, (*Z*)-3-hexenol; 4, α-pinene; 5, β-pinene; 6, myrcene; 7, (*Z*)-3-hexenyl acetate; 8, hexenyl acetate; 9, limonene; 10, (*E*)-β-ocimene; 11, linalool; 12, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 13, (*Z*)-3-hexenyl butyrate; 14, (*E*)-2-hexenyl butyrate; 15, indole; 16, (*Z*)-jasmone; 17, β-caryophyllene; 18, (*E*)-β-farnesene; 19, α-humulene; 20, (*E*,*E*)-α-farnesene; 21, unknown sesquiterpene hydrocarbon; 22, nerolidol; 23, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; 24, unknown sesquiterpene.

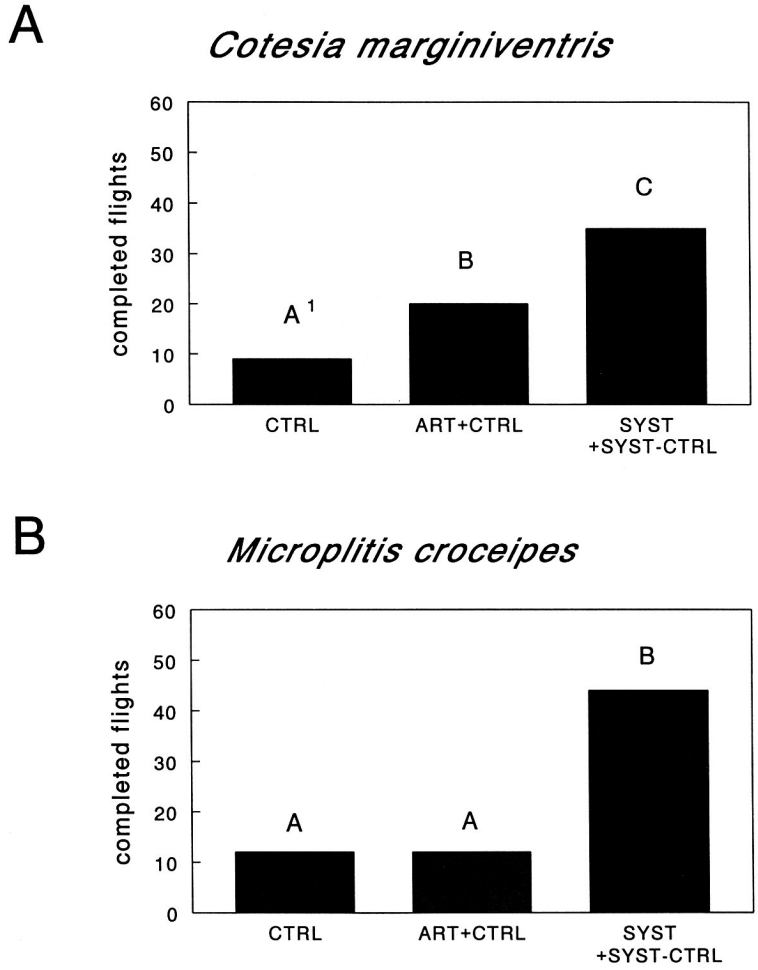

FIG. 2. Comparison of total number of wasps that completed flights to undamaged cotton plants (CTRL), to artificially damaged plants and undamaged plants (ART + CTRL), and to cotton plants that released inducible compounds systemically after feeding damage of beet armyworms on the lower leaves and to undamaged plants, both with the lower leaves removed from the plant (SYST + SYST − CTRL) (A) of the generalist *C. marginiventris* and (B) of the specialist *M. croceipes*. ([1]Fischer's exact test was used to compare the significance of differences in the total number of completed flights to each treatment combination for each wasp species. The behavior was investigated on five different days with a total of $N = 60$ wasps per treatment. Different capital letters above bars indicate significant differences ($P \leq 0.05$).

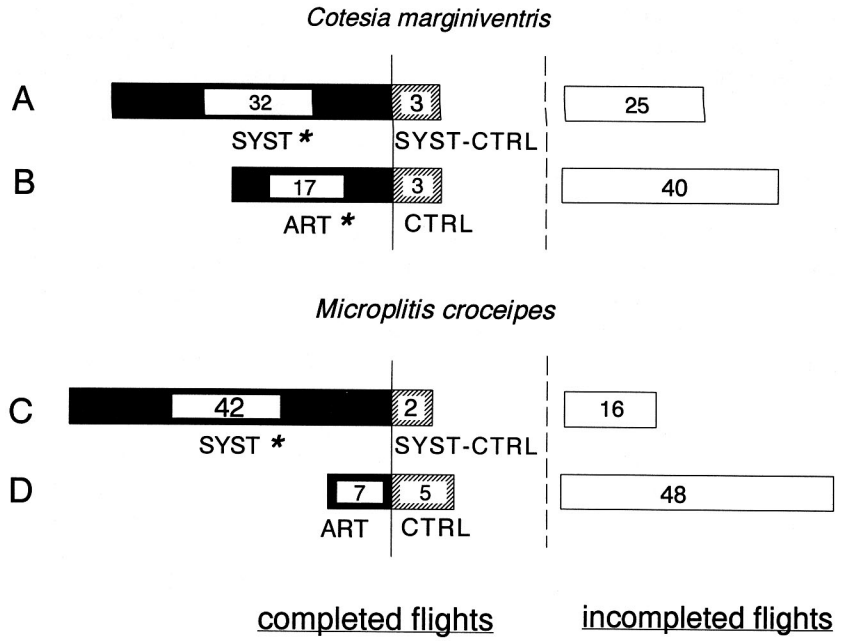

FIG. 3. Flight response of the generalist *C. marginiventris* and the specialist parasitoid *M. croceipes* in two-choice experiments to: (A, C) volatiles released systemically from undamaged leaves of a cotton plant damaged by beet armyworm larvae on the lower leaves (SYST) compared to an undamaged control plant (SYST − CTRL) both with the lower leaves removed; and (B, D) artificially damaged cotton leaves (ART) compared to an undamaged control plant (CTRL). The shaded bars indicate the numbers of wasps that landed on each source, and the open bars show those that did not land on either plant, for each test. Asterisks indicate significant differences in preferences within each pair of odors (Fischer's exact test, $P \leq 0.05$, $N = 60$).

in response to herbivore damage, they are a reliable indication of an herbivore-damaged plant.

Behavioral studies indicated previously that undamaged parts of cassava plants partly damaged by mealybugs were attractive to parasitoids (Nadel and van Alphen, 1987). Furthermore, undamaged parts of spider mite-infested lima beans were attractive to predators (Dicke et al., 1990a,b; Dicke and Dijkman, 1992). Corn seedlings that were partly damaged with a razor blade with caterpillar oral secretion rubbed over the damaged surface were attractive for the parasitoid *C. marginiventris* (Turlings and Tumlinson, 1992). The compounds released systemically by those corn seedlings, in amounts significantly larger than from control seedlings, were (Z)-3-hexenyl acetate and the terpenoids lin-

alool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. All of these previously reported compounds [(*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene] and in addition (*E*,*E*)-α-farnesene and (*E*)-β-farnesene were released from undamaged leaves of caterpillar-damaged cotton plants (Röse et al., 1996) and were also systemically released from cotton plants immediately prior to their use in the flight tunnel for our experiments. It appears that those compounds are not only released from several plant species in response to herbivory, but that those compounds also serve as attractants for a variety of predaceous and parasitic insects. However, since we could only collect volatiles from plants immediately prior to their use in the flight tunnel, but not during the flight-tunnel experiment itself, we can only assume that *M. croceipes* and *C. marginiventris* were responding to those systemically released volatiles.

In our experiments, *M. croceipes* were attracted to plants that released volatiles systemically in response to feeding damage of a nonhost (BAW) on the lower leaves. Our gas chromatographic analysis of systemically released volatiles in response to host (CEW) feeding damage on the lower leaves showed that there were no qualitatively and quantitatively significant differences between systemically released volatiles in response to BAW or CEW feeding on the lower leaves. In addition, some of the inducible compounds released systemically are known to be released from CEW-damaged cotton bolls [(*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene] (Turlings et al., 1993a). The similarities of herbivore inducible compounds systemically released after feeding damage of different herbivore species and also some similarities of volatile compounds released from different feeding sites on the plant may explain the attraction of *M. croceipes* that are naive with regard to cotton volatiles to inducible compounds systemically released after nonhost feeding damage. These findings are in accordance with results obtained from corn plants (Turlings et al., 1993b). When the cut stems of corn seedlings were placed in oral secretion of different lepidopteran species, those corn plants released the same compounds. The parasitoids *C. marginiventris* and *M. croceipes* were attracted to corn plants placed in BAW oral secretion. As in our study, *M. croceipes* were attracted to volatiles systemically released in response to a nonhost. However, with increasing experience of the plant–host complex, small ratio differences in volatile blends may become more important to the parasitoid. Then, wasps may learn to distinguish even between small differences in the blend (Turlings et al., 1993b), whereas the parasitoids in our experiment were naive with regard to cotton plant volatiles.

The specificity of the signal to herbivore damage, but not to the attacking herbivore species, can still be effective, as we previously discussed (Turlings et al., 1995). From the standpoint of the plant, it is not necessarily a disadvan-

tage to attract parasitoids or predators to unsuitable herbivores, as long as the natural enemies of the herbivores are attracted also. Inducible compounds that are systemically released in response to herbivore damage may easily be detected by parasitoids from a distance since those compounds are released in large amounts throughout the entire plant (Röse et al., 1996). This may benefit the herbivore damaged plant by increasing its apparency to beneficial insects. In cotton, these inducible compounds are synthesized *de novo* (Paré and Tumlinson, 1997b). Plant varieties that can respond quickly to herbivore damage by synthesizing and releasing large amounts of inducible compounds may attract beneficial insects faster and thereby minimize herbivore damage. However, from the standpoint of the parasitoid, obviously more specific cues are necessary for the successful location of a host larva. Parasitoids may use nonspecific plant volatiles as a long-range cue to lead them to the general area where they are likely to find a herbivore feeding on a plant. Once they have located such an area, wasps may rely on more specific cues such as host frass (Elzen et al., 1987; Eller et al., 1988; Turlings et al., 1991b; Steinberg et al., 1993; Röse et al., 1997; Cortesero et al., 1997).

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AGGREGATION PHEROMONE OF *Pityogenes knechteli* AND SEMIOCHEMICAL-BASED INTERACTIONS WITH THREE OTHER BARK BEETLES

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Abstract—Gas chromatographic-electroantennographic detection and GC-mass spectrometric analyses of volatile extracts from male and female *Pityogenes knechteli* Swaine identified hexanol, (±)-ipsdienol, and (*S*)-(−)-ipsenol as male-produced candidate pheromone components. In a lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, forest in the southern interior of British Columbia, multiple-funnel traps baited with (±)-ipsdienol alone, (*S*)-(−)-ipsenol alone, or both caught 60%, 6%, and 23%, respectively, of all *P. knechteli* trapped; unbaited traps caught the remaining 11%. In another field trapping experiment, (*S*)-(+)-ipsdienol was as attractive as (±)-ipsdienol, and (*R*)-(−)-ipsdienol was behaviorally benign. (*S*)-(+)-Ipsdienol is thus concluded to be the principal aggregation pheromone component of *P. knechteli*. At low release rates, hexanol increased attraction of beetles to (±)-ipsdienol, or to (±)-ipsidienol plus (*S*)-(−)-ipsenol, but at high release rates hexanol decreased attraction, suggesting a role in preventing overpopulation in the host tree. On the basis of laboratory bioassays in which walking beetles were attracted to (*S*)-(−)-ipsenol, we hypothesize that (*S*)-(−)-ipsenol serves as a short-range attractant for *P. knechteli*. Three sympatric scolytids were also captured in field experiments as follows: the pine engraver, *Ips pini* (Say), to its pheromone (±)-ipsdienol; *I. latidens* LeConte to its pheromone (*S*)-(−)-ipsenol; and *I. mexicanus* (Hopkins), for which the pheromone is unknown, to (*S*)-(−)-ipsenol with (±)-ipsdienol. Although all four species attack lodgepole pine, we have never observed *I. latidens* or *I. mexicanus* attacking the same hosts at *P. knechteli* or *I. pini*. These results suggest that ipsenol and ipsdienol serve as synomones involved in promoting aggregation on the host tree, maintaining species-specific communication, and thus contributing to resource partitioning and reduced competition among the four species.

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Key Words—*Pityogenes knechteli, Ips*, Scolytidae, ipsenol, ipsdienol, pheromone, semiochemical, chirality, interspecific interaction.

INTRODUCTION

Pityogenes knechteli Swaine is a secondary bark beetle inhabiting lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, in British Columbia. It commonly breeds in small-diameter slash and thin-barked portions of trees previously attacked by *Dendroctonus* or *Ips* spp. At high population levels, it may attack small living trees (Bright, 1976; Furniss and Carolin, 1977). There is evidence that male *P. knechteli* produce an aggregation pheromone (Poland and Borden, 1994a), as do males of several related species (Francke, 1977; Baader, 1989).

Other secondary scolytids inhabiting lodgepole pine include *Ips latidens* LeConte, *I. mexicanus* (Hopkins), and *I. pini* (Say). Male *I. pini* produce the pheromone components ipsdienol [with population-specific ratios of enantiomers (Miller et al., 1989, 1996; Seybold et al., 1995)] and lanierone (Teale et al., 1991; Seybold et al., 1992). *Ips latidens* produces ipsenol of unknown chirality, although males showed a slight preference for the (*S*)-(−)-enantiomer (Miller et al., 1991). The pheromone of *I. mexicanus* is unknown, but Miller et al. (1991) captured *I. mexicanus* in traps baited with ipsenol.

In southern British Columbia, *I. latidens* and *I. pini* do not aggregate in the presence of each other's pheromone (Miller, 1991) and do not coinhabit the same host tree (Miller and Borden, 1992). *Ips pini* and *P. knechteli*, in contrast, are less competitive, breed within the same host (Poland and Borden, 1994b), and likely exploit each other's pheromone in host location (Poland and Borden, 1994a).

Our objectives were to identify the aggregation pheromone of *P. knechteli* and to explore which compounds may impart specificity to pheromone communication of *P. knechteli* and three other pine-inhabiting secondary scolytids.

METHODS AND MATERIALS

Experimental Insects. Bolts from lodgepole pines naturally infested with *P. knechteli* were collected in 1993 and 1994 near Princeton, British Columbia and, as needed, transferred into rearing cages held at ~27°C. Emergent beetles were sexed and starved for 24–48 hr on moist filter paper before use.

Collection and Analyses of Volatiles. Approximately 500 male and 500 female *P. knechteli* were confined in gel capsules on separate uninfested logs cut from the same tree. They were allowed to bore into the bark for 24–48 hr and were then excised from the logs and crushed over Dry Ice in a 95 : 5 pentane–

ether solution (10 μl/beetle). The extracts and three synthetic candidate pheromones (hexanol, ipsenol and ipsdienol) were tested for attractiveness in experiments 1–6 (Table 1).

Coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Arn et al., 1975), modified for processing small insects (Gries, 1995), were performed on 10 newly emerged females to identify candidate pheromones in extracts of male beetles. The apparatus used a Varian 3400 gas chromatograph fitted with a DB-5-coated, fused silica column (30 m × 0.32 mm ID; J&W Scientific, Folsom, California). Antennally active compounds were analyzed by coupled GC-mass spectrometry (MS) in electron impact (EI) mode employing a Varian Saturn 3400 ion trap fitted with the same DB-5 column. Synthetic candidate pheromones were subjected to further GC-EAD analyses to compare their EAD activity with those of male-produced compounds. Enantiomeric composition of ipsenol and ipsdienol in volatile extracts was determined by GC-MS (Hewlett Packard 5985B) in selected-ion monitoring (SIM) and chemical ionization (CI, isobutane) mode with a Cyclodex-B column (30 m × 0 .25 mm ID; J&W Scientific). In sequence, synthetic standards, a hexane blank, and concentrated pheromone extract were analyzed.

Laboratory Bioassay Experiments. Volatile extracts and candidate pheromones were tested with walking beetles in a modified open-arena laboratory olfactometer (Wood and Bushing, 1963; Stock and Borden, 1983). Air at 350 ml/min passed through a 10.0-mm-ID glass tube containing filter paper impregnated with test compounds in pentane or pentane alone (control stimulus). In 2-min bioassays, males and females in groups of 10 were released ~10 cm downwind of the tube's exit port. Beetles that entered a 2.0- × 2.0- × 3.0-cm triangular area directly downwind of the exit port were classed as positive responders. Each insect was used no more than twice a day in two different experiments, with a minimum of 2 hr between tests. For any experiment, all replicates were conducted on the same day. To prevent departure of walking beetles, the arena was enclosed with a fine mesh screen, and red lighting was used to minimize spontaneous flight.

Experiment 1 (Table 1) tested the attraction of females to volatile extracts of male and of female beetles. Experiments 2–4 (Table 1) tested the dose-dependent attraction of males and females to (*S*)-(−)-ipsenol (experiment 2), (±)-ipsdienol (experiment 3) and combinations thereof (experiment 4). Experiments 5 and 6 (Table 1) tested (*S*)-(−)-ipsenol plus (±)-ipsdienol alone and in combination with hexanol at low (experiment 5) and high (experiment 6) doses.

Field Trapping Experiments. Experiments 7–12 were conducted in 1994 and 1995 in a lodgepole pine forest 30 km east of Princeton, British Columbia. Twelve-unit multiple-funnel traps (Lindgren, 1983) were placed 15 m apart in

TABLE 1. RESPONSES OF *Pityogenes knechteli* IN LABORATORY BIOASSAYS, EXPERIMENTS 1–6

Experiment	Stimulus	Dose	Percent response[a] Males	Females
1	pentane	10 μl		16.0
	female extract	0.1 BE		12.0
		1.0 BE		20.0
	male extract	0.1 BE		32.0
		1.0 BE		58.0[b]
2	pentane	10 μl	10.0	15.0
	(*S*)-(−)-ipsenol	0.001 ng	12.5	15.0
		0.01 ng	5.0	40.0[b]
		0.1 ng	20.0	30.0
		1.0 ng	37.5[b]	30.0
		10.0 ng	22.5	25.0
		100.0 ng	10.0	7.5
3	pentane	10 μl	17.5	17.5
	(±)-ipsdienol	0.001 ng	37.5[b]	32.5
		0.01 ng	37.5[b]	22.5
		0.1 ng	22.5	37.5[b]
		1.0 ng	30.0	37.5[b]
		10.0 ng	12.5	25.0
		100.0 ng	27.5	17.5
4	pentane	10 μl	17.5	15.0
	(*S*)-(−)-ipsenol	1.0 ng	27.5	20.0
	(*S*)-(−)-ipsenol : (±)-ipsdienol	0.1 : 0.1 ng	25.0	32.5
		1.0 : 0.1 ng	22.5	30.0
		1.0 : 1.0 ng	40.0[b]	37.5[b]
5	pentane	10 μl	15.0	12.5
	(*S*)-(−)-ipsenol : (±)-ipsdienol	1.0 : 1.0 ng	35.0[b]	32.5[b]
	(*S*)-(−)-ipsenol : (±)-ipsdienol : hexanol	1.0 : 1.0 : 0.01 ng	30.0	25.0
		1.0 : 1.0 : 0.1 ng	22.5	25.0
		1.0 : 1.0 : 1.0 ng	22.5	12.5
6	pentane	10 μl	15.0	20.0
	(*S*)-(−)-ipsenol : (±)-ipsdienol	1.0 : 1.0 ng	35.0[b]	47.5[b]
	(*S*)-(−)-ipsenol : (±)-ipsdienol : hexanol	1.0 : 1.0 : 1.0 ng	22.5	30.0
		1.0 : 1.0 : 10 ng	22.5	10.0
		1.0 : 1.0 : 100 ng	27.5	5.0[b]

[a] Five groups of 10 females were tested in experiment 1 and four groups of 10 females and 10 males were tested in experiments 2–6.
[b] Significant difference from response to pentane control, chi-square test, $P < 0.05$.

randomized complete blocks with 10 blocks (replicates) for each experiment. The traps were placed 10–15 m inside the forest margin. All chemicals, sources, release rates, and devices are listed in Table 2. Unbaited control traps were used in all experiments.

Experiment 7 tested the attractiveness of (±)-ipsdienol and hexanol alone and combined. Experiment 8 tested the attractiveness of (±)-ipsenol alone and in combination with (±)-ipsdienol plus hexanol. Experiment 9 tested the attractiveness of hexanol in combination with (*S*)-(+)-, (*R*)-(−)-, or (±)-ipsdienol. Experiment 10 tested the attractiveness of (*S*)-(−)-ipsenol and (±)-ipsdienol alone or in combination. Experiment 11 tested (±)-ipsdienol alone and in combination with hexanol at low, intermediate, and high release rates (Table 2). Finally, Experiment 12 tested (*S*)-(−)-ipsenol plus (±)-ipsdienol alone and in combination with hexanol at low and very low release rates (Table 2).

Statistical Analysis. Data from laboratory bioassays were analyzed by chi-square tests, wherein the response of the pooled test population for each treat-

TABLE 2. DESCRIPTION OF SEMIOCHEMICALS AND METHODS OF THEIR DEPLOYMENT IN THE FIELD, EXPERIMENTS 7–12

Experiment	Semiochemical	Source[a]	Purity[b]	Release device[a]	Release rate (mg/24 hr)
7–12	(±)-ipsdienol	P	97% chemical	bubble cap	0.2[c]
9	(*S*)-(+)-ipsdienol	P	97% chemical 98% optical	bubble cap	0.2[c]
9	(*R*)-(−)-ipsdienol	P	97% chemical 98% optical	bubble cap	0.2[c]
8	(±)-ipsenol	P	99% chemical	bubble cap	0.2[c]
10, 12	(*S*)-(−)-ipsenol	P	96% chemical 97% optical	bubble cap	0.2[c]
12	very low hexanol	S	98%	microcentrifuge tube (250 μl)	0.5[d]
7–9	low hexanol	S	98%	closed polyethylene bottle (15 ml) with hole	3[d]
11, 12	low hexanol	S	98%	bubble cap	3.8[c]
11	intermediate hexanol	S	98%	open polyethylene bottle (15 ml)	15[d]
11	high hexanol	S	98%	open polyethylene bottle with soaked wick	300[d]

[a]Symbols for sources as follows: P = Phero Tech Inc., Delta, British Columbia; S = Sigma Chemical Company, St. Louis. Missouri. Bubble cap release devices manufactured and loaded by Phero Tech.
[b]Purity as listed by manufacturer.
[c]Release rates determined by Phero Tech, Inc. at 22°C.
[d]Determined in laboratory at 24°C.

ment was compared with that of the control population (Stock, 1981). Data from field trapping experiments were analyzed by two-way analysis of variance (ANOVA) using PROC GLM in SAS (SAS Institute, 1994) and the means were compared using the Ryan-Einot-Gabriel-Welsch multiple-range test (REGWF) (Day and Quinn, 1989). If needed, numbers of beetles caught in the traps were transformed to $X' = \log(X + 1)$ to stabilize the variance and normalize the data (Zar, 1984).

RESULTS

Pheromone Analyses. In experiment 1, female *P. knechteli* were highly attracted to a pentane extract of males at a dose of 1.0 beetle equivalents (BE) (Table 1). GC-EAD analyses of this extract revealed three antennally active compounds (Figure 1) that were not detected in volatile extracts from females. GC-MS of these compounds suggested, and GC-MS of authentic standards confirmed, that they were hexanol, ipsenol, and ipsdienol. Synthetic and male-produced compounds at equivalent quantities elicited similar antennal responses.

Analyses of male-produced and synthetic ipsdienol and ipsenol on a chiral Cyclodex B column by GC-MS-CI-SIM resulted in retention time and ion ratio matches of synthetic and male-produced ipsdienol. Both (*S*)-(+)- and (*R*)-(−)-ipsdienol are produced by the beetle (Figure 2). Inconsistent ion ratios of synthetic and male-produced ipsenol may be attributed to an unknown compound coeluting with, and contributing one ion to (*S*)-(−)-ipsenol. Because there was insufficient extract for further analysis, it is not yet confirmed whether male *P. knechteli* indeed produce (*S*)-(−)-ipsenol.

Experiments with Synthetic Volatiles. (*S*)-(−)-Ipsenol attracted both female and male *P. knechteli* in laboratory bioassays, but only at doses of 0.01 and 1.0 ng, respectively (Table 1, Experiment 2). In field trapping experiments, (±)- and (*S*)-(−)-ipsenol failed to attract beetles (Figure 3, experiments 8 and 10, respectively). A blend of (*S*)-(−)-ipsenol with (±)-ipsdienol attracted beetles in laboratory bioassays (Table 1, experiments 4–6) and in the field (Figure 3, experiments 10 and 12), but superior attraction was achieved with (±)-ipsdienol alone (Figure 3, experiment 10). (*S*)-(+)- and (±)-ipsdienol were equally attractive to both males and females, whereas (*R*)-(−)-ipsdienol was behaviorally benign (Figure 3, experiment 9). In experiment 7, the blend of (±)-ipsdienol with hexanol was the only stimulus to result in trap catches (females only) significantly higher than in the unbaited control traps (Figure 3). In experiment 12, hexanol at a very low release rate, in combination with (*S*)-(−)-ipsenol and (±)-ipsdienol, was most attractive, but not significantly so for females (Figure 3). In both the laboratory and the field, high doses of hexanol

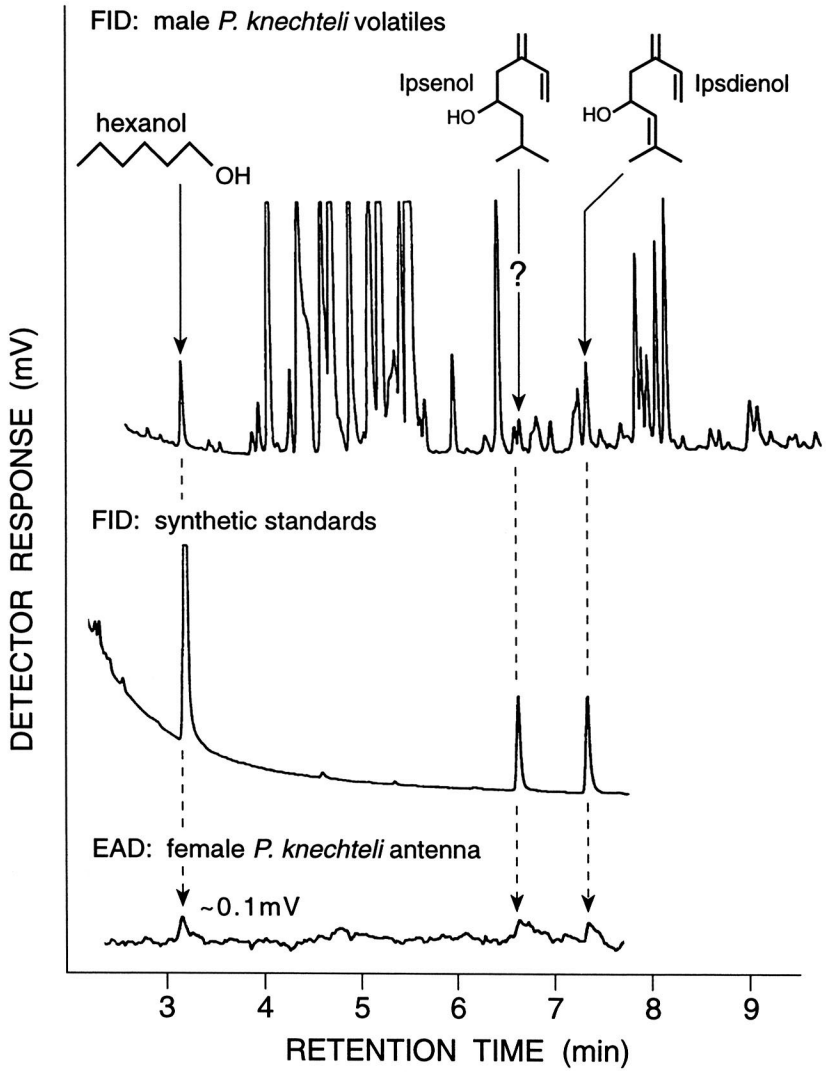

FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD: female *Pityogenes knechteli* antenna) responses to volatile extract from male *P. knechteli* (top) and synthetic standards of hexanol, (±)-ipsdienol, and (±)-ipsenol in a 25:1:1 ratio (middle). Chromatography: DB-5 columns; injector and FID at 240°C; linear flow velocity of carrier gas: 35 ml/sec; temperature program: 1 min at 50°C, 10°C/min to 240°C.

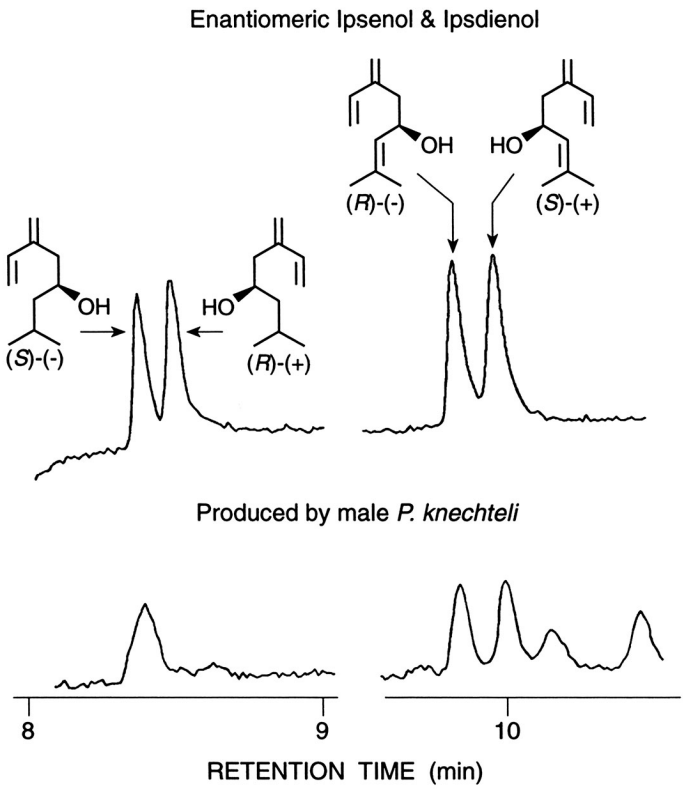

FIG. 2. Selected ion (*m/z* 137 and *m/z* 155 for ipsenol, and *m/z* 135 and *m/z* 136 for ipsdienol) chromatograms (Hewlett Packard 5985 B) of synthetic (top) and beetle-produced (bottom) compounds. Chromatography: Cyclodex-B column; 110°C isothermal; linear flow velocity of carrier gas: 35 cm/sec; injector and FID temperature: 240°C.

caused pronounced reductions in response levels (Table 1, experiments 5, 6; Figure 3, experiment 11).

(±)-Ipsdienol alone or in combination with hexanol attracted *Ips pini* in significant numbers (Table 3, experiments 8–11). (±)- and (*S*)-(−)-Ipsenol reduced attraction of *I. pini* to (±)-ipsdienol (Table 3, experiments 8 and 10). (*S*)-(−)-Ipsenol attracted and (±)-ipsdienol repelled *I. latidens* (Table 3, experiment 10). (*S*)-(−)-Ipsenol alone attracted *I. mexicanus*, but significantly more beetles were captured in traps also baited with (±)-ipsdienol (Table 3, experiment 10). Hexanol did not influence the attraction of *I. pini* to ipsdienol but it did decrease the attaction of *I. mexicanus* to (*S*)-(−)-ipsenol plus (±)-ipsdienol (Table 3, experiment 12).

DISCUSSION

The presence of ipsdienol in volatile extracts of *P. knechteli* males and not of females, the EAD activity of male-produced ipsdienol, and the attraction of both males and females to this alcohol in laboratory bioassays and in field trapping experiments indicate that ipsdienol is a male-produced aggregation pheromone in *P. knechteli*. Although both optical isomers of ipsdienol are produced by males, attraction of conspecifics is associated only with the (*S*)-(+) enantiomer (Figure 3, experiment 9). Evidence indicating that male *P. knechteli* also produce ipsenol includes: (1) presence of an EAD-active compound in GC-EAD recordings coeluting with synthetic ipsenol; (2) presence of fragmentation ions diagnostic of, and coincident with, those of authentic ipsenol in GC-MS analyses; and (3) attraction of both males and females to ipsenol in laboratory bioassays. However, inconsistencies of ion ratios between beetle-produced and synthetic (*S*)-(−)-ipsenol in GC-MS-SIM with a chiral column and lack of attractive characteristics in field experiments support the contention that ipsenol may not be produced by *P. knechteli*. Indeed it has not previously been reported in the genus *Pityogenes*.

We hypothesize that ipsenol is produced in a very low amount and is used as a short-range attractant by *P. knechteli*, accounting for its attractiveness in the laboratory (Table 1). The reduced attractiveness of ipsdienol in the presence of (*S*)-(−)-ipsenol (Figure 3, experiment 10) may be attributed to a disproportionately high release rate of ipsenol. The ratio of ipsenol to ipsdienol may also be critical for optimal attraction of *P. knechteli*. In the genus *Ips*, for example, species-specific communication is achieved, in part, through variations in the enantiomeric composition of ipsdienol, and the relative proportion of ipsenol (Vanderwel and Oehlschlager, 1987). The attraction of the small southern pine engraver, *Ips avulsus* (Eichhoff), the sixspined ips, *I. calligraphus* (Germar), and the eastern fivespined ips, *I. grandicollis* (Eichhoff), to ipsenol and ipsdienol in the field varies with the proportion of each pheromone. A 1:10 ratio of ipsdienol to ipsenol, in combination with *cis*-verbenol, is most attractive to *I. avulsus* and *I. grandicollis*, whereas a 10:1 ratio of the same compounds is most attractive to *I. calligraphus* (Kohnle et al., 1994).

At high release rates, hexanol reduced attraction of *P. knechteli* to ipsdienol in the field (Figure 3, experiment 11) and to a combination of ipsdienol and ipsenol in laboratory bioassays (Table 1, experiments 5 and 6). Because at a very low dose it enhanced attraction (Figure 3, experiment 7), it may be classed as a multifunctional pheromone that at low doses promotes aggregation of conspecifics in a newly attacked host, but at high doses serves as an epideictic pheromone (Prokopy, 1981) that prevents overpopulation. A similar duality in function is attributed to 3-methyl-2-cyclohexen-1-one in the Douglas fir beetle, *Dendroctonus pseudotsugae* Hopkins (Rudinsky, 1973). Hexanol was also iden-

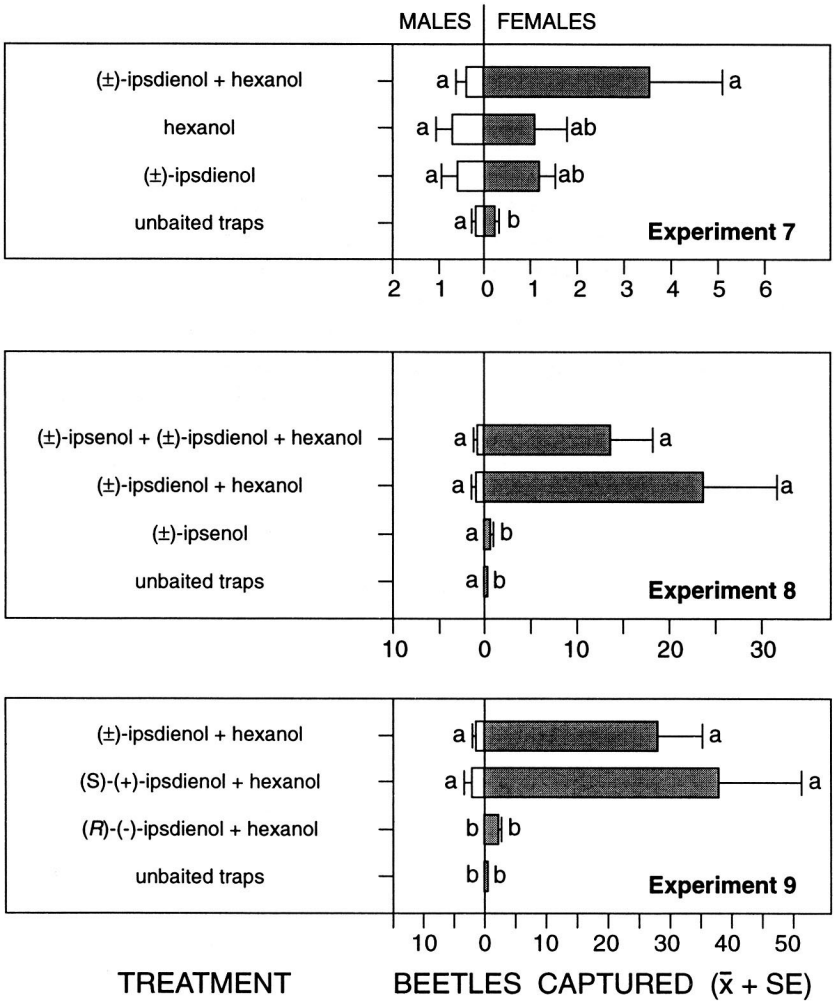

FIG. 3. Numbers of *Pityogenes knechteli* caught in multiple funnel traps in a lodgepole pine forest near Princeton, British Columbia (experiment 7: April 29–May 15, 1994; experiment 8: Aug. 20–Sept. 10, 1994; experiment 9: Aug. 20–Sept. 10, 1994; experiment 10: July 24–Sept. 6, 1995; Experiment 11: Aug. 20–Sept. 10, 1994; Experiment 12: July 24–Sept. 6, 1995). Within each experiment, bars with the same letter for each sex are not significantly different, two-way ANOVA and REGWF test on data transformed by $X' = \log(X + 1)$, $P < 0.05$.

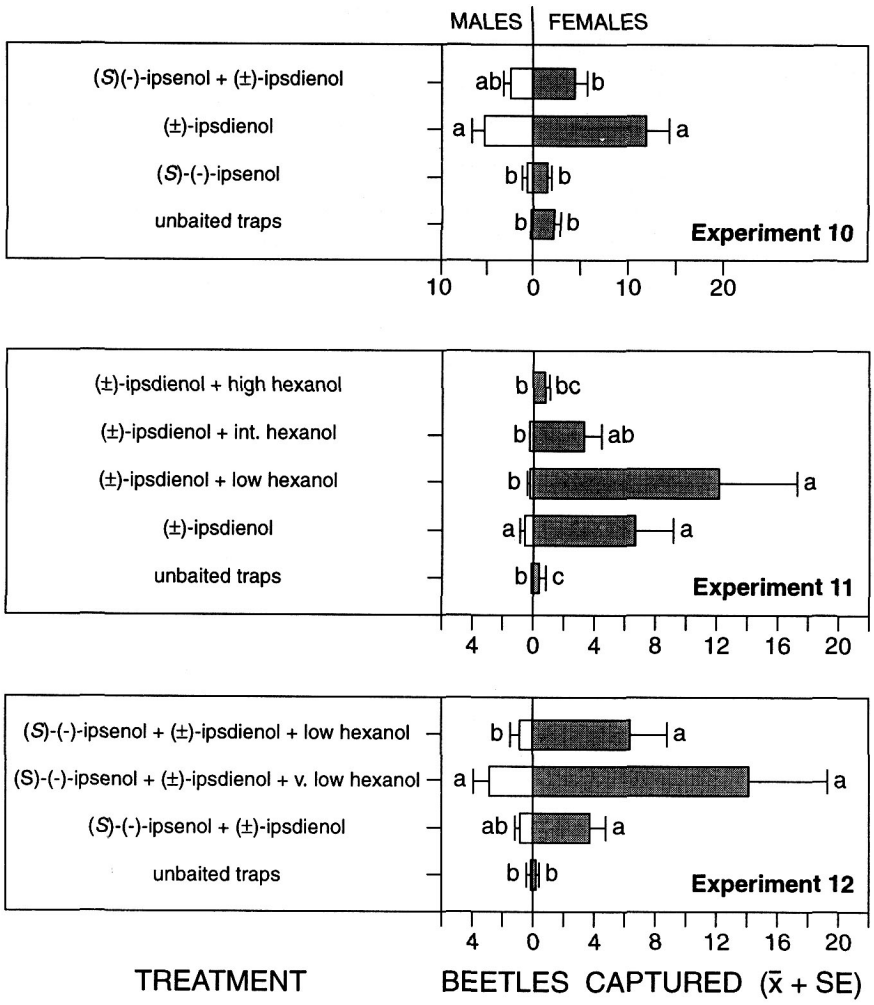

FIG. 3. Continued.

tified as a male-specific peak in *P. chalcographus* L. (Francke, 1977) and *P. quadridens* Hartig (Francke et al., 1995). Baader (1989) reported that hexanol reduced the response of *P. chalcographus* to its aggregation pheromone, but Francke (1977) observed no apparent effect.

Pityogenes knechteli, *I. pini*, *I. latidens*, and *I. mexicanus* all coinhabit the lodgepole pine forest and compete for the available phloem resource. When the

TABLE 3. CATCHES OF ASSOCIATED BARK BEETLES IN EXPERIMENTS 8–12 IN MULTIPLE FUNNEL TRAPS BAITED WITH CANDIDATE PHEROMONES FOR *Pityogenes knechteli*[a]

Exp.	Species	Stimulus	Number of beetles captured (mean ± SE)[b] Males	Females
8	*Ips pini*	Unbaited control traps	0.1 ± 0.1a	0.3 ± 0.3b
		(±)-ipsenol	0.1 ± 0.1a	0 ± 0b
		(±)-ipsdienol + hexanol	0.3 ± 0.2a	4.0 ± 1.4a
		(±)-ipsdienol + hexanol + (±)-ipsenol	0.1 ± 0.1a	0.7 ± 0.4b
9	*Ips pini*	Unbaited control traps	0 ± 0b	0 ± 0c
		(*R*)-(−)-ipsdienol + hexanol	0.2 ± 0.2b	0.9 ± 0.3b
		(*S*)-(+)-ipsdienol + hexanol	0.4 ± 0.2b	0.1 ± 0.1c
		(±)-ipsdienol + hexanol	2.5 ± 0.5a	7.5 ± 1.8a
10	*Ips pini*	Unbaited control traps	0.1 ± 0.1b	0.3 ± 0.2b
		(*S*)-(−)-ipsenol	0.1 ± 0.1b	0.2 ± 0.1b
		(±)-ipsdienol	13.1 ± 3.4a	45.0 ± 10.9a
		(*S*)-(−)-ipsenol + (±)-ipsdienol	0.2 ± 0.2b	1.7 ± 0.8b
	Ips latidens	Unbaited control traps	0.2 ± 0.1b	0.2 ± 0.1b
		(*S*)-(−)-ipsenol	9.2 ± 2.7a	14.2 ± 5.0a
		(±)-ipsdienol	0.3 ± 0.2b	0.4 ± 0.2b
		(*S*)-(−)-ipsenol + (±)-ipsdienol	1.0 ± 0.4b	0.3 ± 0.2b
	Ips mexicanus	Unbaited control traps	0.1 ± 0.1c	0.1 ± 0.1c
		(*S*)-(−)-ipsenol	7.4 ± 1.2b	10.0 ± 1.7b
		(±)-ipsdienol	0.4 ± 0.3c	0.9 ± 0.4c
		(*S*)-(−)-ipsenol + (±)-ipsdienol	10.9 ± 2.0a	26.6 ± 3.7a
11	*Ips pini*	Unbaited control traps	0 ± 0b	0 ± 0b
		(±)-ipsdienol	3.3 ± 0.6a	10.7 ± 2.2a
		(±)-ipsdienol + low hexanol	3.3 ± 1.4a	12.8 ± 4.0a
		(±)-ipsdienol + int. hexanol	2.3 ± 0.6a	6.2 ± 1.6a
		(±)-ipsdienol + high hexanol	2.0 ± 0.5a	5.8 ± 1.1a
12	*Ips mexicanus*	Unbaited control traps	0 ± 0c	0.1 ± 0.1c
		(*S*)-(−)-ipsenol + (±)-ipsdienol	10.1 ± 2.1a	31.9 ± 6.6a
		(*S*)-(−)-ipsenol + (±)-ipsdienol + very low hexanol	11.3 ± 2.6a	28.3 ± 5.7a
		(*S*)-(−)-ipsenol + (±)-ipsdienol + low hexanol	4.9 ± 1.0b	14.7 ± 3.9b

[a]Table 1 for release devices and rates.
[b]Means within an experiment followed by the same letter are not significantly different, REGWF test, $P < 0.05$.

response of all four species to ipsenol and ipsdienol is compared (Figure 4), it appears that the two terpene alcohol pheromones are interacting to maintain species-specific communication and to partition host resources.

Pityogenes knechteli and *I. pini* often coexist within the same host, partitioning the phloem resource mostly on the basis of tree diameter (Poland and Borden, 1994b). *Ips pini* is more aggressive than *P. knechteli* and will colonize healthier trees. Ipsdienol is the main pheromone of both species (Figure 5, no. 1), accounting for cross-attraction of *P. knechteli* to *I. pini*-infested bolts (Poland and Borden, 1994a). Because *P. knechteli* produces very low amounts of its pheromones, the presence of ipsenol in the pheromone blend would probably not inhibit *I. pini* from responding to a host colonized by *P. knechteli* but might deter it at close range from superimposing its galleries on those of *P. knechteli*. This would explain the observation that *I. pini* showed no attraction to *P. knechteli*-infested logs, but the presence of *P. knechteli* did not decrease the attraction of *I. pini* to logs infested by both species (Poland and Borden, 1994a).

In the field, we have observed coattack by *I. latidens* and *I. mexicanus* in trees unsuccessfully attacked by the mountain pine beetle. *Ips latidens* uses ipsenol as an aggregation pheromone (Miller et al., 1991), and our results suggest that both ipsenol and ipsdienol may be part of the pheromone blend for *I. mexicanus*, with ipsenol as the main component (Figure 4). Cross-attraction of *I. mexicanus* to *I. latidens* pheromone, ipsenol, is thus possible (Figure 5, no. 2). Neither *I. latidens* nor *I. mexicanus* have been recorded, or found by us, to breed in the same host as *I. pini* or *P. knechteli*. The repellency of both *P. knechteli* and *I. pini* by ipsenol in the field would thus allow them to detect and avoid hosts colonized by their competitors *I. latidens* and *I. mexicanus* (Figure 5, nos. 3–6). Similarly, ipsdienol produced by *I. pini* and *P. knechteli* would serve as a synomone, deterring the approach of *I. latidens* (Figure 5, nos. 3 and 5). Ipsdienol, ipsenol, and hexanol may therefore play an important role for *P. knechteli* in partitioning host resources with competing species such as *I. latidens* and *I. mexicanus*.

We suggest that the pheromone produced by *P. knechteli* is not the most important cue used by the beetles in location of host material. During the two years of this study, trap catches have always been extremely low in the spring, whereas a relatively large number of beetles were trapped in late summer–early fall. Thus, in the spring, when fresh uninfested host material is abundant and before the emergence of *I. pini*, *P. knechteli* might rely more heavily on host volatiles such as β-phellandrene (Miller and Borden, 1990) or ethanol (Klimetzek et al., 1986) than on pheromone to locate suitable hosts. Later in the season, however, when fresh uninfested hosts are less abundant and *I. pini* is also seeking the same type of breeding material, *P. knechteli* probably uses mostly ipsdienol as a long-range attractant and, being a less aggressive species than

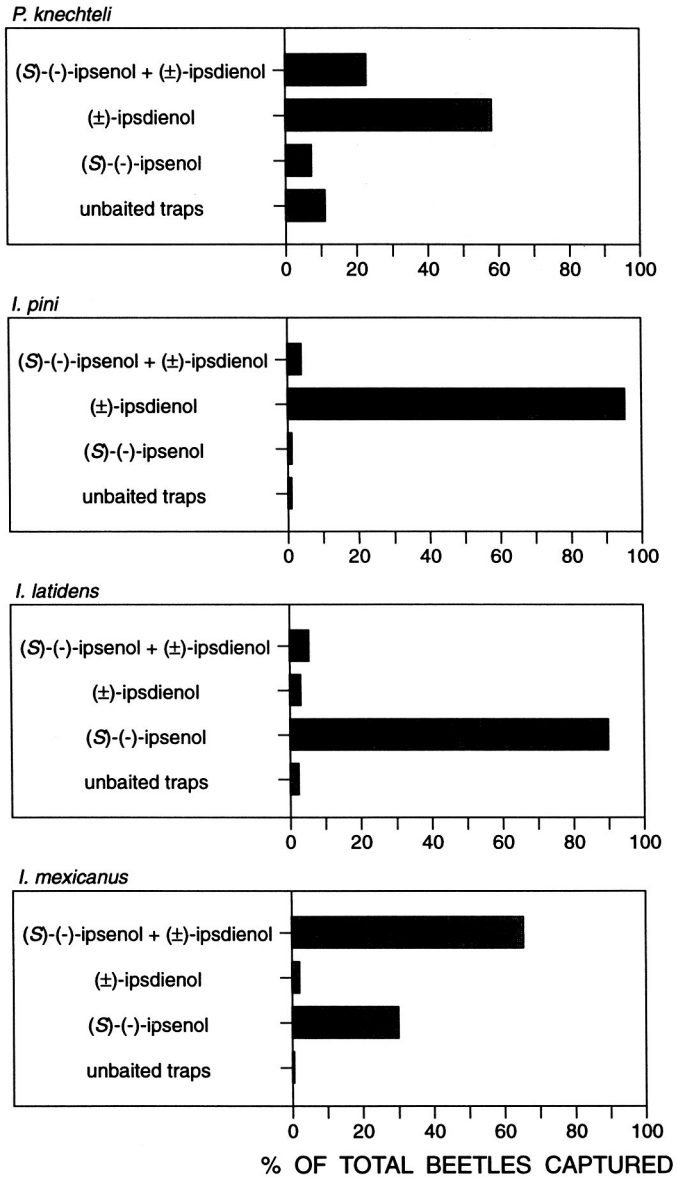

FIG. 4. Comparative responses in experiment 10 of *Pityogenes knechteli*, *Ips pini*, *I. latidens*, and *I. mexicanus* to pheromones produced by male *P. knechteli*. Data normalized for clarity. Similarity of response by sex (Figure 4, Table 3) justifies pooling of data.

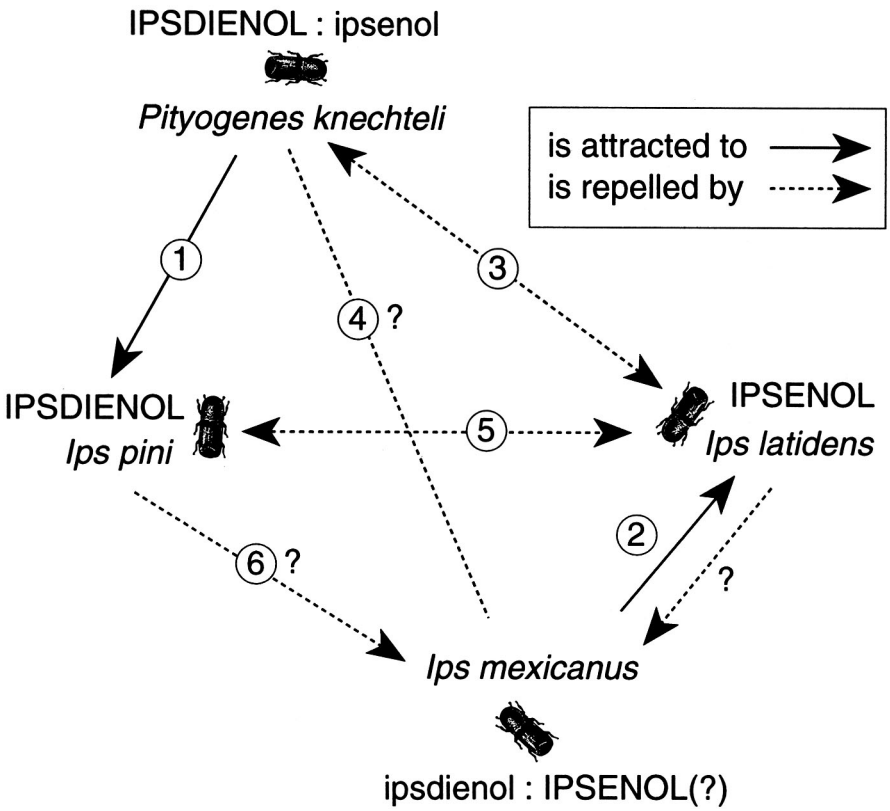

FIG. 5. Diagram depicting the interactions (circled numbers) between *Pityogenes knechteli*, *Ipi pini*, *I. latidens*, and *I. mexicanus*.

I. pini, exploits the pheromone produced by *I. pini* as an aid in host location. Similarly, *I. grandicollis*, the least aggressive of the five cohabiting species in southern pines, shows the most cross-attraction to the other species' pheromones, which may facilitate its ability to locate and occupy the wide range of host conditions used by the other species (Smith et al., 1990).

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DAMAGE-INDUCED ACCUMULATION OF PHYTOECDYSTEROIDS IN SPINACH: A RAPID ROOT RESPONSE INVOLVING THE OCTADECANOIC ACID PATHWAY

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Abstract—Some plant defenses are known to be rapidly induced following attack by phytophagous insects. Plant-produced insect molting hormones, termed phytoecdysteroids, are believed to aid plant resistance; however, their dynamics are poorly understood. Using spinach (*Spinacia oleracea*) as a model system, we examined the inducibility of phytoecdysteroids, primarily 20-hydroxyecdysone (20E), in an effort to characterize potential interactions with herbivorous insects. Rapid phytochemical induction was investigated using damage treatments and applications of defense-related plant-signal analogs, specifically methyl jasmonate (MJ) and methyl salicylate (MSA). Within two days, mechanically damaged roots exhibited two to three fold increases in phytoecdysteroid concentrations. Four days after root damage, small increases in shoot levels were also detectable. Unlike roots, foliar 20E concentrations were unaltered over a range of shoot treatments including insect herbivory (*Spodoptera exigua*), mechanical damage, and MJ applications. Additions of MJ (12.5–50 μg/liter) to the root systems of hydroponically grown plants stimulated accumulations of root phytoecdysteroids in a dose-dependent manner, similar in magnitude to the response induced by root damage. Under identical conditions, MSA did not affect the accumulation of 20E when added to the hydroponic solutions of undamaged plants. Moreover, MSA inhibited the induction of 20E in wounded roots, but did not interfere with the action

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of applied MJ. In contrast to mechanical damage, roots did not induce 20E levels when challenged with two different fungal pathogens (*Pythium aphanidermatum* and *Phytophthora capsici*). We propose that wound-induced accumulations of 20E are generated in the roots, signaled via endogenous jasmonates, and may confer enhanced resistance against subterranean herbivorous insects.

Key Words—Spinach, *Spinacia oleracea*, induced defense, 20-hydroxyecdysone, phytoecdysteroid, roots, methyl jasmonate, methyl salicylate, *Pythium aphanidermatum*, *Phytophthora capsici*.

INTRODUCTION

Soon after the structural elucidation of the first insect molting hormone (Huber and Hoppe, 1965), active analogs were detected in plants (Nakanishi et al., 1966; Galbraith and Horn, 1966). These plant-derived steroids with molting hormone activity are termed phytoecdysteroids (PEs). Over the past 30 years, advances have been made in both the understanding of ecdysteroid-regulated insect physiology and the methodology of their separation, isolation, and identification (reviewed by Koolman, 1989). As a result, over 250 different ecdysteroids have been identified, with the vast majority coming from plant families (Lafont and Horn, 1989; Lafont, 1997). 20-Hydroxyecdysone (20E) is commonly detected PE in plants and is considered to be the major endogenous molting hormone in insects (Bergamasco and Horn, 1983; Rees, 1989). Despite significant progress in ecdysteroid research, little is known concerning the mechanistic and functional roles of PEs in plants.

Phytoecdysteroids are widely distributed among classes of vascular plants (Bergamasco and Horn, 1983). Their patchy systematic distribution is the basis for the argument that they cannot function as general plant growth hormones (Sláma, 1979; Lafont et al., 1991). Attempts to demonstrate a plant hormonal role for PEs have been unconvincing (Carlisle et al., 1963; Felippe, 1980). The only reproducible effect of exogenous PEs on plants is a weak gibberellin-like activity in dwarf mung bean and rice bioassays (Dreier and Tower, 1988; Machácková et al., 1995). Hendrix and Jones (1972), however, failed to find any response to 20E in four different types of gibberellin bioassay. Investigations of PE involvement in flower development have also failed to uncover significant effects (Jacobs and Suthers, 1971; Heftman, 1975; Machácková et al., 1995).

More evidence exists in favor of the hypothesis that PEs confer resistance against nonadapted phytophagous insects. Many studies have shown that when incorporated into artificial diets, PEs can disrupt the insect molting cycle and in turn cause elevated mortality (Robbins et al., 1970; Singh and Russell, 1980; Kubo et al., 1983; Lafont et al., 1991). However, few intact plant bioassays have demonstrated that effects on herbivorous insects are due primarily to the

presence of PEs and not to a combination of factors attributed to complex plant chemistries (Arnault and Slama, 1986; however, see Melé et al., 1992). Often, only subtle responses, such as feeding deterrency, have been demonstrated in PE-adapted herbivorous insects (Kubo and Klocke, 1983; Tanaka et al., 1994). Not surprisingly, some insect species have efficient PE detoxification mechanisms and are highly resistant to dietary PEs (Modde et al., 1984; Zhang and Kubo, 1993; Blackford et al., 1996).

Plants are like insects insofar as both actively respond to a wide range of stimuli (Silvertown and Gordon, 1989). Many plants produce enhanced levels of toxic or deterrent metabolites following herbivory and mechanical damage (reviewed by Baldwin, 1994), and these induced responses often deter subsequent insect herbivory (Karban and Myers, 1989; Tallamy and Raupp, 1991). The need for increased agricultural productivity has prompted investigations of resistance mechanisms already present in crop plants. Towards this goal, there is now emphasis on the elucidation of signal transduction pathways that stimulate plant defense mechanisms against insects and pathogens (reviews Enyedi et al., 1992; Ryan and Jagendorf, 1995).

Jasmonic acid (JA) and salicylic acid (SA) are two endogenous signals implicated in eliciting plant resistance responses. JA is produced following cell damage (i.e., herbivory) via the octadecanoid pathway (Farmer and Ryan, 1992; Hamberg and Gardner, 1992). Induced accumulations of JA activate a variety of insect and microbial defense responses in plants. A few well-known JA-inducible products include proteinase inhibitors, benzophenanthridine alkaloids, nicotine, phenolics, and flavonoids (Farmer and Ryan, 1990; Mueller et al., 1993; Baldwin et al., 1997; Mizukami et al., 1993; Gundlach et al., 1992). JA is thought to initiate these changes by modulating both defense and regulatory gene expression (Farmer et al., 1992, Gundlach et al., 1992; Choi et al., 1994). Unlike JA, SA-based defense signaling involves primarily disease resistance responses (Klessig and Malamy, 1994). SA is believed to stimulate fungal and viral resistance, in part, by inducing pathogenesis-related proteins (Ward et al., 1991). While important in some defense responses, SA and related analogs actually block JA biosynthesis. Thus, SA is used to investigate both pathogen resistance responses and effects caused by inhibiting wound-induced JA biosynthesis (Peña-Cortés et al., 1993). Despite much recent research, the precise responses induced by endogenous JA and SA are only well understood for a few plant systems.

The regulation of PE accumulation in whole plants has received little attention to date (Adler and Grebenok, 1997). Lafont et al. (1991) reflected on the potential inducibility of PEs following insect or nematode attack and implied that increased production, in response to damage, would reflect a plant defense function. The empirical demonstration of enhanced levels of PEs following damage and MJ treatments would lend support to the theory that PEs function

as plant defenses. Patterns of PE biosynthesis, transport, and accumulation have been established in spinach, thus creating an ideal model system for investigating the potential inducibility of these compounds in higher plants (Grebenok and Adler, 1991; Grebenok et al., 1991). Using this system, our study addresses the following questions: (1) Do either root or shoot tissues display induced accumulations of PEs following damage? (2) Do known plant defense signals stimulate altered patterns of PE accumulation? (3) Is there a candidate signaling pathway for the induction of 20E? (4) Is there evidence for 20E induction specificity under different types of tissue attack?

METHODS AND MATERIALS

Chemicals. Methyl salicylate (MSA), (±)-methyl jasmonate (MJ), lanolin, and all plant nutrient components were purchased from the Sigma Chemical Co. (St. Louis, Missouri). HPLC grade solvents were acquired from the Fisher Chemical company (Pittsburgh, Pennsylvania).

Plant Growth. Plants were reared in an environmental chamber at 22°C, with a 12L:12D cycle and uncontrolled humidity. Fluorescent lighting supplied approximately 145 μmol photons/sec/m^2 of photosynthetically active radiation (PAR) at the leaf surface. Spinach (*Spinacia oleracea* var. Avon) seeds from W. Atlee Burpee Co. (Warminster, Pennsylvania) were germinated in vermiculite and grown for 8–12 days, then transplanted into individual plastic cups (1-liter) and grown hydroponically for an additional 10–15 days. The hydroponic solution was modified from Hoagland and Arnon (1950) and contained 0.236 g/liter $Ca(NO_3)_2 \cdot 4H_2O$, 0.101 g/liter KNO_3, 0.016 g/liter NH_4NO_3, 0.493 g/liter $MgSO_4 \cdot 7H_2O$, 0.068 g/liter KH_2PO_4, and 0.0459 g/liter FeNa-EDTA. The micronutrients included 3.09 mg/liter H_3BO_3, 1.979 mg/liter $MnCl_2 \cdot 4H_2O$, 0.288 mg/liter $ZnSO_4 \cdot 7H_2O$, 0.0624 mg/liter $CuSO_4 \cdot 5H_2O$, and 0.0968 mg/liter $Na_2MoO_4 \cdot 2H_2O$. One day prior to each experiment, plants of uniform morphology and size were selected and weighed. Treatment groups, with similar mean fresh masses, were formed by sorting plants based upon fresh mass and assigning plants to groups by consecutive divisions (see Ohnmeiss and Baldwin, 1994). At the start of each experiment, plants were randomly assigned growth-chamber shelf positions. No additional nutrients were added to the solutions before or during the experiments. Solution levels were maintained by the addition of distilled water as needed.

Phytoecdysteroid Quantification. After the treatment and response period (two to four days), plants were dissected into roots and shoots, frozen, and lyophilized to dryness. Each tissue sample was ground to a fine powder using a Wiley mill (850-μm mesh; Thomas Scientific), and a weighed portion (normally 50–60 mg) was extracted in 10 ml methanol for 48 hr. An 7-ml aliquot

was mixed with 3 ml H_2O, and partitioned against 10 ml hexane. Two days after phase separation, 8 ml of the aqueous methanol layer was removed and evaporated to dryness. The residue was resolubilized in 5 ml H_2O and partitioned against 5 ml butanol. A 4-ml aliquot of the butanol phase was evaporated and resuspended in 200 μl methanol prior to analysis. For quantitative purposes, all partitions utilized previously countersaturated solvents. Reverse-phase high-performance liquid chromatography (RP-HPLC) of spinach PEs was carried out on a C-18 column (Alltech, Spherisorb ODS-2, 4.6 × 150 mm, 5 μm). Early experiments used an isocratic 9:11 methanol/water solvent system delivered at 1.0 ml/min at 35°C (Grebenok et al., 1991). This protocol does not separate 20E from polypodine B and thus provides a measure only of total PEs. To achieve PE separation, subsequent experiments utilized an isocratic 2-propanol–water (12:88) mobile phase under the same conditions (Morgan and Marco, 1990). The second solvent system confirmed polypodine B to be a minor component (<7.0%) of the previously combined PE peaks. The plant-derived HPLC peak coeluting with the pure 20E standards was identified as 20E by direct exposure probe chemical ionization mass spectrometry (M. F. Feldlaufer, personal communication). For each experiment, PE quantification was extrapolated from concurrently generated external standard curves, constructed by independent weightings of 20E. Correction factors were applied to account for ecdysteroids lost throughout the sample partitioning and injection processes. Single-step extractions and solvent partitions were found to consistently recover greater than 80% of the total PEs that could be obtained using multiple steps (Schmelz, unpublished).

Screen for Phytoecdysteroid Induction. A thorough search for phytochemical inducibility should include treatment and analysis of both roots and shoots because of different responses often detected in these tissues (Zangerl and Rutledge, 1996). Comparisons between real and simulated herbivory should also be performed because the temporal, spatial, and oral nature of insect feeding often produces plant responses different from mechanical damage alone (Baldwin, 1990; Alborn et al., 1997). To this end, we examined the effects of shoot mechanical damage (SD), shoot herbivory by beet armyworms (BAWs), root mechanical damage (RD), root removal (RR), and combined RD and SD treatments on PE concentrations. Two MJ levels also were tested due to the ability of jasmonic acid to systemically induce plant defenses in other systems (Farmer et al., 1992; Baldwin et al., 1994).

Independent measurements were taken at two time points for each of the seven treatment groups. This involved destructively harvesting individual plants either two or four days after the experiment was initiated. Including the zero-, two-, and four-day controls, this experiment utilized 17 groups ($N = 5$) of plants having an initial wet mass of 1.01 ± 0.02 g (grand mean ± SEM).

SD was accomplished by rolling a pattern-tracing wheel (Dritz Co., Spar-

tanburg, South Carolina) across the first two fully expanded leaves (see Baldwin et al., 1997). Each leaf received six rows of damage parallel to the mid-rib, with three of the rows on each side spaced approximately 0.5 cm apart. The average total distance across the leaf surface covered by this wheel was 23 cm and resulted in approximately 100 punctures per plant. The punctures were generated by the spokes of the tracing wheel rolling across the surface, which was supported underneath by a plastic card. Individual punctures resulted in 1-mm^2 zones of cell death.

BAW was achieved by confining five early third-instar *Spodoptera exigua* to each plant. Armyworms were removed after 18 hours on the plants. On average, this treatment resulted in the removal of 15% of the total leaf area, with all leaves receiving some damage.

Root damage consisted of either RD or RR. The RD treatment was created by firmly squeezing sections of root between the thumb and index finger. This process produced an area of damage approximately 2 cm long, and was repeated five times to distribute the damage equally along the length of the root (25 cm). Cells in the damaged area exhibited some browning (i.e., cell death), but, since roots continued to grow, transport processes appeared to be largely intact. The dual treatment of RD and SD combined the two previously described treatments. The RR treatment involved the removal of the distal 20% of the root system by mechanically shearing via a rubbing motion between the thumb and index finger. Latex gloves were used in all root damage treatments in an attempt to avoid unnecessary microbial introductions. The two MJ treatments involved the addition of 5.0 ml aqueous aliquots containing 205 and 390 μg of MJ to the hydroponic solutions. The MJ additions created initial hydroponic solution concentrations of 0.92 and 1.74 μM, respectively.

Phytoecdysteroid Induction Sensitivity to Root Applied MJ. If jasmonates are important endogenous signals in the induction of root PEs, then activity should be detectable using relevant signal molecule concentrations. We examined a range of MJ concentrations to determine a sensitivity threshold for the induction of 20E. Forty plants were separated into eight treatment groups (N = 5, 0.828 $\pm$ 0.013 g) consisting of seven MJ concentrations and one control group. Separate stock solutions of aqueous MJ, created through independent weighings, were introduced into the individual hydroponic 1-liter solutions in amounts of 2.5, 6, 12.5, 25, 50, 100, and 200 μg/plant. These MJ additions resulted in final concentrations of 11, 27, 56, 112, 223, 446, and 893 nM MJ, respectively. Plants were harvested 72 hr after the start of the experiment.

Phytoecdysteroid Induction Sensitivity to Shoot Applied MJ. Previous experiments indicated that roots, but not shoots, respond to direct tissue damage by increasing 20E levels. The physiological basis for uninducible shoot PEs following leaf damage might involve either a lack of PE pathway receptivity to wound signals, and/or deficient endogenous wound signal production. Using a

range of foliar MJ treatments with known systemic activity in alkaloid induction (Baldwin et al., 1994), we addressed whether shoots contain a PE induction-pathway that is receptive to an applied MJ signal. Twenty-four plants were assigned to six treatment groups (N = 4, 2.257 ± 0.066 g): three foliar MJ concentrations in lanolin paste, one root MJ concentration, a lanolin paste control, and an untreated control. MJ was applied to the leaf surfaces in amounts of 50, 200, or 500 μg, carried by a lanolin paste (average mass = 0.093 g/plant). Each lanolin droplet was divided between the four fully expanded leaves of a given plant and was spread among the basal third of the upper leaf surface. Plants were harvested four days after the start of the experiment. Leaf sections containing the lanolin paste (30% of each treated leaf) were included in dry mass calculations, but were not analyzed for 20E.

Manipulation of Octadecanoic Acid Pathway. Investigations of damage-induced responses triggered by the octadecanoic acid pathway have been aided by the use of inhibitors (Peña-Cortés et al., 1993). MSA is known to inhibit the octadecanoic acid pathway (Baldwin et al., 1996, 1997). We designed experiments to determine whether MSA can block the induction of 20E caused by RD, MJ, and RD + MJ treatments. Our hypothesis was that the octadecanoic acid pathway is required for the damage-induced accumulation of PEs in spinach roots. MSA was used to test the following predictions: (1) If damage produces endogenous jasmonate signals that trigger PE induction, then MSA should inhibit this response. (2) Because MSA is believed to prevent only the production of the JA signal, MSA should not interfere with the actions of applied MJ. (3) The inhibition of PE induction in damaged roots, caused by MSA, should be reversible with the addition of MJ.

The interactive effects of RD, MJ, and MSA on 20E accumulation patterns were investigated by dividing 32 plants into the following eight treatment groups (N = 4, 1.427 ± 0.038 g): (1) an untreated control. (2) RD, (3) hydroponic MJ addition, (4) RD + MJ, (5) hydroponic MSA addition, (6) MSA + RD, (7) MSA + MJ, and (8) MSA + RD + MJ. Preliminary experiments demonstrated that a wide range of hydroponic MSA concentrations (5–5000 μg/liter) did not affect root or shoot 20E accumulation patterns in undamaged plants. However, MSA concentrations as low as 50 μg/liter provided significant inhibition of root PE accumulation in RD plants. All treatments involving aqueous MJ and MSA resulted in final hydroponic solution concentrations of 50 μg/liter. Successful inhibitor action required infiltration of the target site. To aid this process, all treatments that involved MSA were performed 6 hr prior to the RD treatment and MJ application. The RD treatment was as previously described, except that only three damage sites were created instead of five. Plants were harvested 72 hr after completion of the last treatment.

Effect of Fungal Pathogens on 20E Pools. A variety of physical and biotic factors are known to cause root damage. We investigated the ability of spinach

plants to differentiate between wound signals caused by root-infesting fungal pathogens (*Pythium aphanidermatum* and *Phytophthora capsici*) and those derived from mechanical damage (i.e., jasmonic acid). If the induction of root 20E pools is targeted against below-ground insect herbivores, then plants might display some specificity in the induction of this response. To test this, 16 plants were divided into four treatment groups (1.54 $\pm$ 0.06 g), which included one untreated control, one positive control for MJ root induction, and two fungal infection treatments. Fungal inoculation consisted of an 8-mm-diameter disk (V8 agar) excised from the advancing margin of a 5-day-old culture of the isolated pathogens (Stanghellini et al., 1996). A single agar disk was placed in the hydroponic solution of each fungal inoculated plant. Pathogen-free V8 agar disks were placed in the untreated controls and the root MJ (200 μg/plant) treated positive controls. To avoid fungal contamination of the main growth chamber, the experiment was performed in a smaller isolated chamber. Plants were incubated under an 18L:6D 23°C/18°C cycle, and an average PAR of 100 μmol/sec/m^2. Plants were harvested for analysis on the fourth day, at which time those experiencing the *P. aphanidermatum* treatment showed characteristic browning of the entire root system. Likewise, plants challenged with *P. capsici* displayed root lesions at the air–water interface. Spinach is a known host for the pathogen *P. aphanidermatum* but has not been previously described as a host for *P. capsici* (Larsson, 1992).

Statistical Analysis. Analyses of variance (ANOVAs) were performed on root and shoot measurements for all experiments. Specific treatment effects were investigated, if the main effects of the ANOVAs were significant ($P < 0.05$). In the first two experiments, Dunnett's tests (one-tailed) were used for defined and limited contrasts where increases above the control were of primary interest (Zar, 1996). The third and fifth experiments utilized Tukey tests to correct for multiple comparisons where no a priori hypothesis existed (Wilkinson and Hill, 1994a). In the fourth experiment (dealing with the MSA inhibition of damage induced 20E), protected contrasts were utilized to test a limited number of specific a priori hypothesis. In this experiment, Bonferroni corrections were employed for the six contrasts which lowered the accepted alpha from 0.05 to 0.0083 (Wilkinson and Hill, 1994b, p. 265). Data analysis was accomplished with the aid of the MGLH module of the SYSTAT statistical package (Evanston, Illinois).

RESULTS

Damage-Induced Accumulation of Root Phytoecdysteroids. A rapid induction of root PEs was observed both two and four days after root damage and

root MJ additions. The induction of shoot PEs occurred on day 4, but only with treatments involving root damage.

On day 2, treatments that caused significant increases (ANOVA $F_{7,32} = 13.44$, $P < 0.001$) in root PEs included: root damage (RD, 2.87-fold), root damage plus shoot damage (RD + DS, 2.07-fold), root removal (RR, 1.77-fold), MJ 205 μg/liter (2.12-fold), and MJ 390 μg/liter (2.07-fold) (Dunnett test $P < 0.025$; Figure 1B–D). Treatments using exclusively either shoot herbivory by the BAW or SD did not significantly increase root PE concentrations (Dunnett test $P > 0.490$). In stark contrast to the roots, no statistically significant differences (ANOVA $F_{7,32} = 1.73$, $P = 0.137$) were detected in shoot PE concentrations at day two (Figure 1A–D).

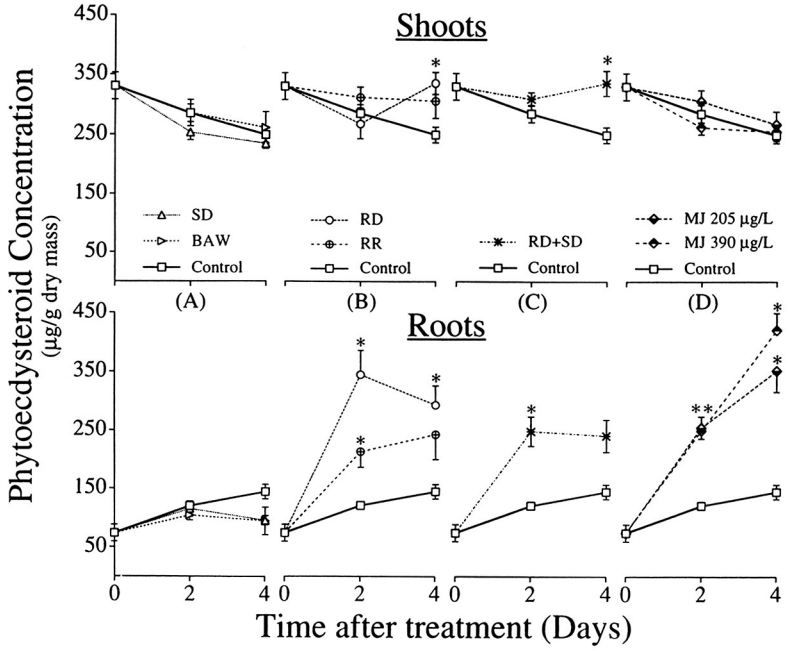

FIG. 1. Mean (±SEM) root and shoot phytoecdysteroid concentrations following treatments of root mechanical damage (RD), root removal (RR), shoot mechanical damage (SD), beet armyworm leaf-herbivory (BAW), and root plus shoot mechanical damage (RD + SD). Also included are two separate methyl jasmonate (MJ) treatments consisting of either 205 or 390 μg/liter in the hydroponic solutions. Untreated control groups are redrawn to aid in visual comparisons. Asterisks denote significant increases above the control group, $P < 0.05$, using Dunnett's test for defined multiple comparisons. SEMs are small and obscured by plot symbols at some harvest times.

On day 4, significant differences (ANOVA $F_{7,32} = 16.79$, $P < 0.001$) persisted in root PE accumulation patterns. Significant increases were maintained after treatments of RD (2.03-fold), 205 μg/liter MJ (2.44-fold), and 390 μg/liter MJ (2.92-fold) (Dunnett test $P < 0.003$; Figure 1B and D). Treatments of RR and RD + SD demonstrated mean increases in root PEs (1.7-fold above the controls); however, this effect was only marginally significant (Dunnett test $P < 0.062$). On day 4, foliar damage caused by the BAW and SD treatments had no effect (Dunnett test $P > 0.360$) on root PE concentrations (Figure 1A).

Alterations in shoot PE concentrations were manifested on day 4 (ANOVA $F_{7,32} = 3.895$, $P < 0.004$). Root damage initiated by the RD and RD + SD treatments caused significant (Dunnett test $P < 0.015$) 1.34-fold increases in shoot PE concentrations (Figure 1B, C). All other treatments, including BAW and SD, failed to change the shoot PE accumulation patterns (Dunnett test $P > 0.120$). Over the four-day experiment, root and shoot biomass increased 1.8- and 2.0-fold for each respective tissue type. All treatment groups demonstrated identical increases in shoot (ANOVA $F_{7,32} = 0.27$, $P > 0.960$) and root dry biomass (ANOVA $F_{7,32} = 1.68$, $P > 0.150$). Thus, treatment induced differences in PE concentrations were independent of effects on plant biomass.

Nanomolar Concentrations of MJ Induce Root PEs. MJ demonstrated activity in the initial screen for PE induction; thus, we investigated whether MJ might function at relevant signal molecule concentrations. In hydroponically grown spinach, MJ concentrations as low as 56 nM were sufficient to induce root PE concentrations (Figure 2). Additions of aqueous MJ to the hydroponic solution caused significant increases (ANOVA $F_{7,32} = 13.50$, $P < 0.001$) in roots, but not in shoots (ANOVA $F_{7,32} = 0.59$, $P = 0.758$). MJ additions of 12.5 μg/liter (56 nM) or higher induced significant increases (Dunnett test $P < 0.050$; Figure 2) in root PE concentrations, MJ treatments of 12.5, 25, and 50 μg (representing 56, 112, and 223 nM MJ in the final hydroponic solution) resulted in 1.8-, 2.3-, and 3.1-fold increases in root PE concentrations, respectively. For the three-day experimental interval, MJ concentrations above 50 μg/liter did not cause further increases. Root and shoot dry biomass measures were not significantly altered (ANOVA $F_{7,32} = 0.59$, $P > 0.759$) by any of the treatments.

Tissue-Specific Responses to MJ. Many mechanisms could prevent shoots from undergoing a PE induction response after foliar damage. We compared the effects of root and shoot MJ applications on PE induction responses to differentiate between deficient wound-signal production and a lack of PE pathway receptivity in the presence of wound signals. Specifically, we examined whether shoots contain a PE induction pathway that is receptive to an applied MJ signal. Applications of MJ to the spinach leaves did not alter the 20E concentrations in any tissues; thus, the shoot PE pathway appears insensitive to jasmonates.

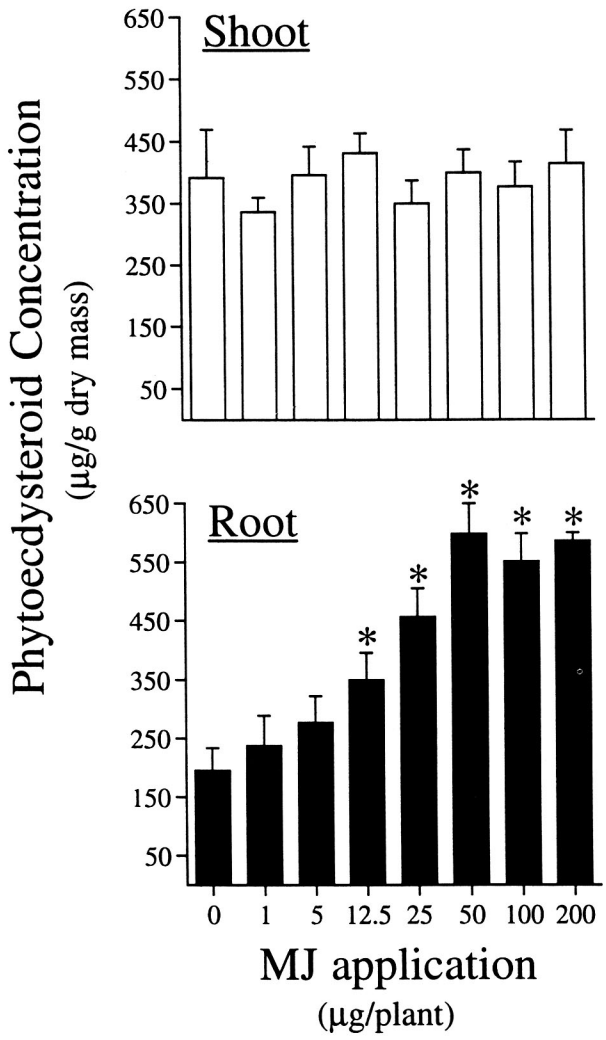

FIG. 2. Mean (±SEM) root and shoot phytoecdysteroid concentrations three days after hydroponic methyl jasmonate (MJ) additions of 1, 5, 12.5, 25, 50, 100, and 200 μg/plant. These additions represent final hydroponic solution concentrations of 11, 27, 56, 112, 223, 446, and 893 nM MJ, respectively. Asterisks denote significant increases above of the control group, $P < 0.05$, using Dunnett's test for defined multiple comparisons.

While no significant differences were found in shoot 20E concentrations, differences (ANOVA $F_{1,18} = 21.68$, $P < 0.001$) were detected in the roots (Figure 3). Hydroponic additions of 200 μg/plant MJ, representing a positive control for plant inducibility, stimulated a significant (2.86-fold) increase over the lanolin control. As observed for shoots, root 20E concentrations were not significantly affected by foliar treatments of pure lanolin or MJ in lanolin.

Inhibition and Recovery of 20E Inducibility. We used MSA, an octadecanoic acid pathway inhibitor, to address the following questions: (1) Does MSA inhibit the damage induced accumulation of root 20E? (2) Can applied MJ still function in the presence of the MSA inhibitor? (3) Can MJ recover the induction response of damaged roots in the presence of the MSA inhibitor?

Hydroponic additions of MSA inhibit the wound-induced accumulations of root 20E. Significant differences (ANOVA $F_{7,24} = 12.55$, $P < 0.001$; Figure 4) were detected in root 20E concentrations. Root damage significantly increased (protected contrast $F_{1,24} = 11.54$, $P = 0.002$) root 20E concentrations above the untreated control, MSA control, and the RD + MSA groups combined. However, in the presence of MSA, root damage did not (protected contrast $F_{1,24} = 0.04$, $P = 0.841$) induce 20E levels above the untreated and MSA control groups (Figure 4).

Unlike damage, the action of applied MJ was not inhibited in the presence of MSA. Combined MJ + MSA treatments produced a significant 20E induction above the untreated and MSA controls (protected contrast $F_{1,24} = 11.87$, $P = 0.002$) and did not differ (protected contrast $F_{1,24} = 0.89$, $P = 0.354$) from the treatment with MJ alone. The MSA inhibition of damage-induced 20E accumulation was recoverable by MJ additions. In the presence of MJ, the RD + MSA treatment produced more 20E than the untreated control, the MSA control, and the RD + MSA groups combined (protected contrast $F_{1,24} = 51.53$, $P < 0.001$; Figure 4). Moreover, this RD + MSA + MJ treatment did not differ (protected contrast $F_{1,24} = 0.01$, $P = 0.986$) from the RD + MJ treatment alone.

Shoot 20E concentrations typically averaged 180 μg/g dry mass and did not significantly differ (ANOVA $F_{7,24} = 1.72$, $P = 0.152$). Thus, for purposes of graphical simplification, the shoot 20E data are not presented. As in the previous experiments, final root or shoot dry biomass measures were not different (ANOVA $F_{7,24} = 0.88$, $P > 0.541$).

Specificity in Elicitation of Induced 20E. We investigated whether spinach plants can differentiate between mechanical damage signals (i.e., JA) and fungal pathogen attack in an effort to determine the potential specificity of the induced 20E response. Total pool sizes of root 20E differed significantly (ANOVA $F_{3,12} = 43.45$, $P < 0.001$) between treatment groups. However, root inoculations with fungal pathogens *Phytophthora capsici* and *Pythium aphanidermatum* did

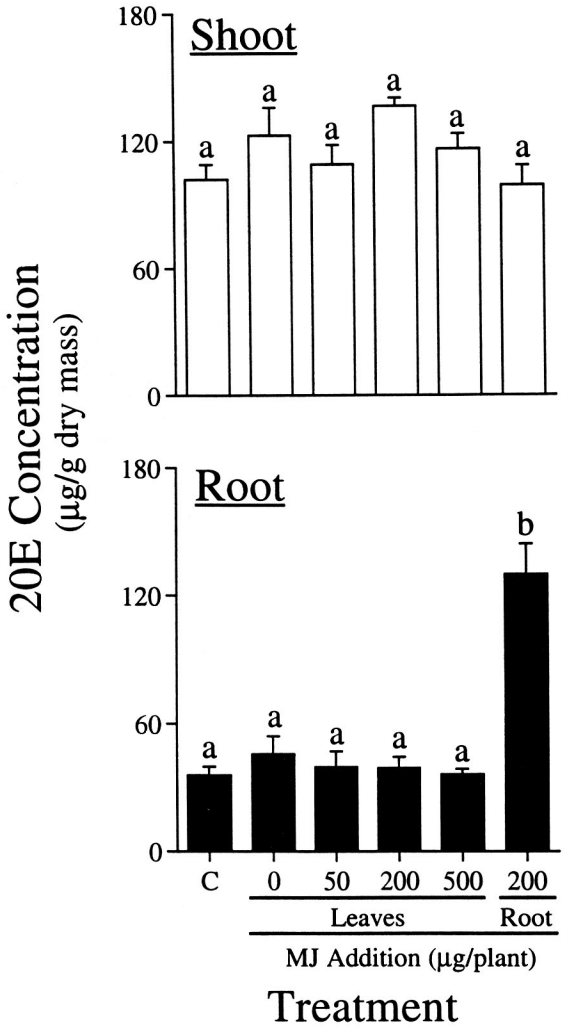

FIG. 3. Mean (±SEM) root and shoot 20-hydroxyecdysone (20E) concentrations four days after foliar methyl jasmonate (MJ) applications in a lanolin paste. Shoot applications of thinly coated lanolin (0.093 g divided between four leaves) resulted in either 0, 50, 200, or 500 μg of MJ/plant. The aqueous MJ treatment of the root (200 μg/plant) also contained pure shoot lanolin, whereas the true control (C) remained untreated. Within root and shoot plots, columns bearing different letters are significantly different ($P < 0.05$, Tukey correction for multiple comparisons).

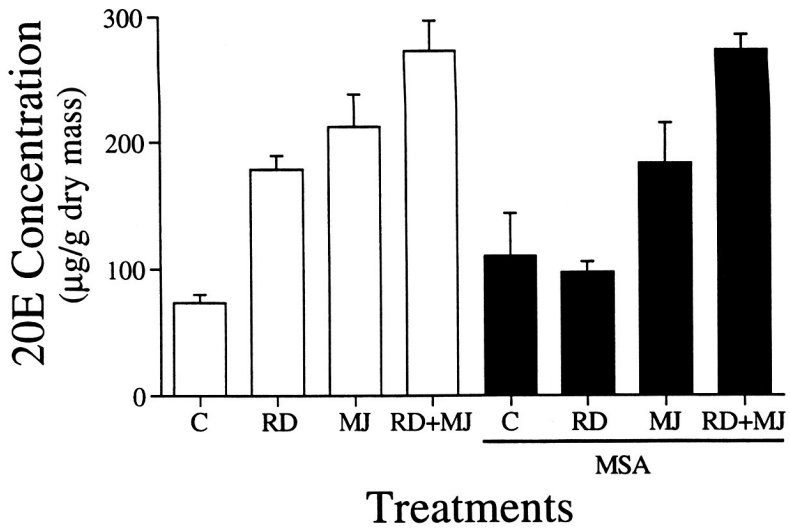

FIG. 4. Mean (±SEM) root 20E concentrations three days after treatments involving; untreated controls (C), mechanical root damage (RD), methyl jasmonate (MJ), and RD + MJ. In a near identical set of four groups of plants, methyl salicylate (MSA) was added to the hydroponic solution 6 hr prior to the four primary treatments. All additions of MSA and MJ resulted in 50 μg of compound/plant in the individual 1-liter hydroponic solutions. Dark and white bars represent MSA-treated and untreated plants, respectively.

not elicit increases in the root accumulation of 20E pools (Figure 5). The hydroponic addition of MJ (200 μg/liter), employed as a positive control for inducibility, stimulated 3.1- to 4.6-fold increases in root 20E accumulation, as compared to all other groups. No significant differences (ANOVA $F_{3,12} = 2.78$, $P > 0.086$) were found between treatment groups in root or shoot dry biomass measures.

DISCUSSION

The results of our experiments indicate the following. First, root PE concentrations can be rapidly (48–72 hr) induced two- to threefold by root damage and MJ applications, whereas shoot PEs only show small increases four days after root damage. Second, MJ stimulates PE accumulation at relevant signal molecule concentrations (nanomolar levels) and acts in a dose-dependent manner. Third, increases in root 20E induced by mechanical damage are inhibited by additions of MSA; however, MJ can override the effects of MSA and restore

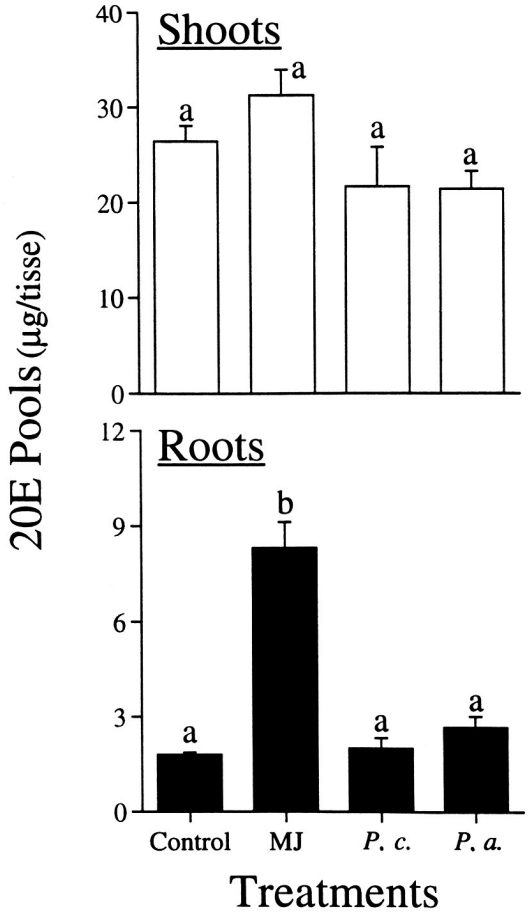

FIG. 5. Root and shoot 20E pools (mean ± SEM) four days after aqueous additions of methyl jasmonate (MJ) 200 μg/plant, and root inoculations with the fungal pathogens *Phytophthora capsici* (P. c.) and *Pythium aphanidermatum* (P. a.). Within root and shoot plots, columns bearing different letters are significantly different ($P < 0.05$, Tukey correction for multiple comparisons).

the inducibility of inhibited plants. This supports an involvement of endogenous jasmonates in inducible PE accumulation. Fourth, in contrast to root treatments involving mechanical damage and MJ, fungal pathogen inoculation does not induce 20E accumulation. The rapidity of the responses, coupled to a general lack of altered tissue biomass, suggests that the induction of PEs is an active response and not simply the result of changes in plant growth.

Root damage and root MJ additions cause similar responses in both the timing and magnitude of induction on day 2. On day 4, the PE response to root mechanical damage waned, which is consistent with the transient nature of a damage-induced JA signal (Baldwin et al., 1994, 1997). At the same time, MJ treatments continued to stimulate increases in root 20E accumulation, likely reflecting the continued presence of MJ in the hydroponic solutions. Unlike the situation in roots, the PE biosynthetic pathway in shoots appears to be largely unresponsive to shoot mechanical damage and insect folivory. While ridged shoot PE accumulation patterns follow leaf damage and MJ treatments, increases in shoot PEs can be detected four days after root damage. Thus, while the induction responses caused by RD and root MJ appear similar, differences do exist. Additional types of wound signals elicited by mechanical damage may account for these observed differences. Shoot MJ applications (50–500 μg) failed to induce either root or shoot 20E levels. This suggests both a lack of receptivity in the shoot PE pathway to jasmonates and an inability of jasmonates to function systemically in spinach. This contrasts with the situation in tomato and tobacco, where MJ induces systemic increases in plant defenses, regardless of the site of application (Farmer et al., 1992; Baldwin et al., 1994).

SA signaling in plants is normally associated with pathogen attack (Klessig and Malamy, 1994). Our preliminary experiments demonstrated that MSA does not stimulate the induction of root PEs at concentrations ranging from 5 to 5000 μg/liter (Schmelz, unpublished). Endogenous production of JA is catalyzed by a series of enzymes in the octadecanoid pathway. SA and related analogs, including acetylsalicylic acid and MSA, are believed to inhibit the octadecanoid pathway leading to JA by interfering with the cyclooxygenase step (Peña-Cortés et al., 1993; Baldwin et al., 1996, 1997). In tomato, Peña-Cortés et al. (1993) demonstrated that aspirin inhibits both the wound-induced accumulation of JA and the subsequent induction of proteinase inhibitor II. Only octadecanoid metabolites subsequent to the cyclization step were able to restore proteinase inhibitor II inducibility in the presence of aspirin.

In spinach, MSA additions do not affect the MJ-induced accumulations of root 20E. However, low levels (50 μg/liter) are sufficient to inhibit the wound-induced accumulations of root PEs. The MSA inhibition of damage-induced PE accumulation is reversible by addition of exogenous MJ, as would be predicted for a JA-signaled response. Our results are consistent with the SA-based suppression of endogenous JA production; the inhibitory action of SA is ineffective against octadecanoid metabolites that have completed the cyclization step. Moreover, MJ additions as low as 12.5 μg/liter (56 nM) are sufficient to induce root PE concentrations. This exogenous level is within the range of induced endogenous JA concentrations (Farmer, 1994) and compares favorably with MJ sensitivity in other systems (Gundlach et al., 1992; Farmer et al., 1992; Baldwin

et al., 1994). Thus, we propose jasmonic acid as a candidate signal for the induction of 20E in spinach.

Taken as a whole, our results suggest that induced accumulations of 20E are caused by active root processses. Spinach leaves are known sites of PE biosynthesis, but root synthesis has also been established in other systems (Grebenok and Adler, 1993; Tomás, et al., 1993). While the true mechanism responsible for generating induced accumulations of root PEs is currently unknown, the modulation of gene expression and de novo synthesis has been predicted (Gundlach et al., 1992; Creelman et al., 1992) and is currently under investigation.

Preliminary experiments indicated that root PE levels are extremely sensitive to mechanical perturbation. However, root damage caused by the fungal pathogens *Phytophthora capsici* and *Pythium aphanidermatum* did not induce the root accumulation of 20E pools. This lack of root PE induction was somewhat surprising based on the sensitivity of spinach roots to mechanical damage and the fact that jasmonate-induced responses are often elicited by a wide range of stimuli. In tomato, proteinase inhibitors I and II are induced by plant derived oligouronide fragments, fungal cell wall fragments, wounding, and heat. All of these elicitors are thought to act through the JA pathway (Ryan, 1992; Peña-Cortés et al., 1995). Unlike proteinase inhibitor induction in tomato, PE induction may require specific stimuli as suggested in other systems. For example, in *Solanum tuberosum*, discrimination between wound and pathogen signals involves the production of either steroid-glycoalkaloids or sesquiterpenoid phytoalexins (Choi et al., 1994). From a defense standpoint, induction of PEs only makes sense in a limited number of situations, primarily those involving insect herbivory. The inability of fungal pathogens to induce root PE accumulation supports the idea that plants may be able to differentiate between types of attacking organisms and mount appropriate defenses.

We currently do not know which subterranean herbivorous insects induce root PEs in spinach. Known root pests of chenopodiaceous crops include flea beetles (Coleoptera: Chrysomelidae), wireworms (Coleoptera: Elateridae), root maggots (Diptera: Otitidae), and cutworms (Lepidoptera: Noctuidae) (Whitney and Duffus, 1986; Cooke and Dewar, 1992; Rubatzky and Yamaguchi, 1997). Extensive documentation of root-feeding insect pests does exist for table and sugar beets (*Beta* spp.), which are close relatives of spinach (Jones and Dunning, 1969; Whitney and Duffus, 1986). A moderate level of continuity exists between the host ranges of insects that feed on chenopodiaceous crops. Many members of the plant family Chenopodiaceae are reported to harbor identical species of aphids, thrips, cutworms, leafhoppers, and spider mites (Lange, 1987); thus, numerous insects may be causing the induction of root 20E in the field.

The effectiveness of induced 20E levels in deterring subsequent herbivory

will undoubtedly be context specific, with regards to both the insect species and development stage. When ingested by nonadapted insect herbivores, 20E can cause complete inhibition of growth and molting abnormalities, leading to death (Kubo et al., 1983). Dietary 20E is also known to stimulate deterrent receptors (Tanaka et al., 1994) and inhibit feeding behavior (Jones and Firn, 1978; Blackford and Dinan, 1997) in some phytophagous insects. However, numerous insects can metabolize PEs (Zhang and Kubo, 1993; Blackford et al., 1996) and may be unaffected by increased PE levels. Despite recent advances (Blackford and Dinan, 1997), the effects of PEs on a given insect herbivore are known for only a handful of species. Thus, predictions concerning PE interactions with previously untested species are tenuous. It should be noted that in addition to PEs, the presence of phenolics, oxalates, and triterpenoid saponins in spinach may also influence the observed plant–insect interactions (Huang et al., 1986; Libert and Franceschi, 1987; Tschesche et al., 1969).

To our knowledge, this study represents the first demonstration of an ecologically relevant stress altering PE accumulation patterns in any plant. Rapid root induction opens the possibility of an induced defense in spinach against rhizosphere-dwelling insect herbivores. Even though crop yield losses of greater than 50% are often caused by root herbivory, investigations of root–herbivore interactions have been largely ignored (Brown and Gange, 1990). Addressing the functional significance of root PE induction will require a multifaceted approach. Future research will focus on plant responses to root herbivory by insects and the actual mechanism of induced PE accumulation in spinach.

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ACTIVITY OF ENANTIOMERS OF SULCATOL ON APTERAE OF *Rhopalosiphum padi*

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Abstract—GC-MS analysis of volatiles released from wheat infested with a high density of aphids showed the presence of 6-methyl-5-hepten-2-ol (sulcatol). The proportion of enantiomers present in the volatiles was determined by esterifying the mixture with (1*S*)-(−)-camphanic chloride and quantifying the esters. The mixture consisted of 75% (*R*)-(−) and 25% (*S*)-(+). The mixture of enantiomers as well as the racemate showed significant repellency towards apterous *Rhopalosiphum padi* in an olfactometer (15.7% and 14.4%, respectively, with 10 ng of stimulus). Single enantiomers or a mixture containing 25% (*R*)-(−)- and 75% (*S*)-(+)-enantiomers were inactive. The results are discussed in relation to the achievement of specificity by aphids in different pheromone-mediated behaviors.

Key Words—Sulcatol, aphid, spacing pheromone, enantiomeric synergism.

INTRODUCTION

Many intraspecific chemical signals have been involved in processes related to population dynamics of insects such as reproduction, spacing, aggregation, and defense (Silverstein, 1984; Löfstedt, 1991; Pickett et al., 1992). Among the mechanisms developed by insects to attain species specificity through these chemical signals are: differences in chemical structure, constitutional isomerism, stereoisomerism, different ratios in multiple component pheromone complexes, and chiral isomerism (Silverstein, 1984; Mori, 1989; Seybold, 1993). In most cases of chiral isomerism, the naturally occurring enantiomer is much more active than the antipode (Dickens and Mori, 1989; Tengö et al., 1990). Never-

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theless, the antipode may inhibit the response to the naturally occurring enantiomer (Pierce et al., 1987; Camacho et al., 1993). Furthermore, enantiomeric synergism has been shown when both enantiomers are naturally occurring (Borden et al., 1976; Millar et al., 1985; Oehlschlager et al., 1987, 1988).

Enantiomers of sulcatol (6-methyl-5-hepten-2-ol), an aggregation pheromone of the ambrosia beetle *Gnathotrichus sulcatus* (Byrne et al., 1974), have been shown to exhibit enantiomeric synergism. Borden et al. (1976) showed that *G. sulcatus* responded to a broad range of mixtures of enantiomers of sulcatol including the racemate, while *Gnathotrichus retesus* (Le Conte) produced only the *S* enantiomer and did not respond to the racemate (Borden et al., 1980). Recently, Quiroz et al. (1997) found that the naturally occurring mixture of 6-methyl-5-hepten-2-one, (−)- and (+)-sulcatol, and 2-tridecanone released from wheat seedlings heavily infested with aphids (9 aphids/cm^2) was repellent to apterous *Rhopalosiphum padi* (Homoptera: Aphididae) in olfactometric assays and produced population deaggregation of aphids feeding on wheat. Furthermore, the ketones in the natural mixture were found individually to produce repellency in the olfactometer (Quiroz et al., 1997). In this paper, we report the use of (1*S*)-(−)-camphanic chloride to resolve a racemic mixture of sulcatol. We also report the effect of single enantiomers and mixtures on apterae of *R. padi.*

METHODS AND MATERIALS

Aphids. Colonies of *R. padi* were started with individuals collected in grass fields near the Facultad de Ciencias, Universidad de Chile, and kept on oat (*Avena sativa* L. cv. Nahuén) in a growth room at 18–22°C and a light regime of 18L:6D.

Olfactometry. We used a common bioassay for aphids (Campbell et al., 1993; Pettersson et al., 1994, 1995) in which an aphid placed in a Perspex olfactometer can be exposed to volatiles coming from containers attached to each of its four side arms (two opposed containers contain the treatment, the other two the control). The observation area is divided into four arm zones and one central indifferent zone. The time the aphid spends in each arm is registered during 15 min. Double solvent and blank control showed no bias in the olfactometer. Pseudoreplication (Hurlbert, 1984) was avoided by changing the treatment and control stimuli and the test aphid and by disassembling and cleaning the olfactometer after each repetition. Each experiment was replicated 10 times, and results were analyzed using Wilcoxon's one-tailed rank-sum test for two groups).

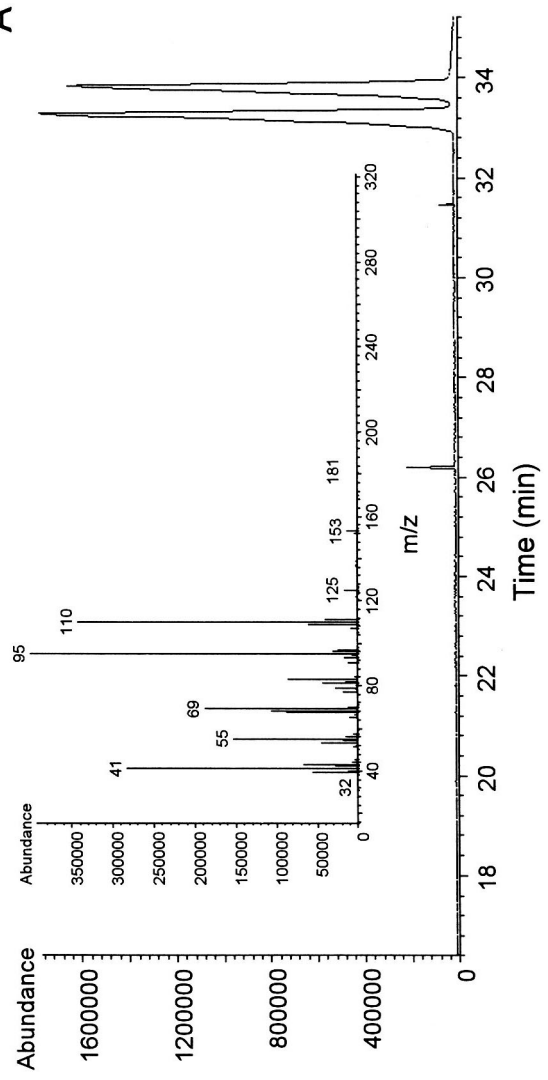
Capture and Analysis of Volatiles. Twenty wheat seedlings in decimal

growth stage 20 (Zadoks et al., 1974) infested with apterae of *R. padi* (ca. 1500) at high density (ca. 9 aphids/cm^2) were placed in bell jars permeated by air dried and purified by passage through activated 5 Å molecular sieves and charcoal. The air was drawn at 1 l/min for 48 hr, and the volatiles captured on Porapak Q (Blight, 1990). The volatiles were desorbed from the Porapak with 2.5 ml of freshly distilled diethyl ether, and the extract was concentrated to 50 μl under a stream of nitrogen. Aliquots (1 μl) of the concentrated extract were used in GC-MS analysis on a capillary α-DEX 120 Supelco GLC column (25 m × 0.2 mm ID) directly coupled to a mass detector and an integrated data system (GC model HP-5890, MD model HP-5972). Ionization was by electron impact at 70 eV and 280°C. The GC oven was maintained at 40°C for 6 min, then programmed to increase at 15°C/min to 150°C, then 10°C/min to 200°C, and finally 0.1°C/min to 220°C.

Preparation of Diasteroisomeric Esters of (±)-Sulcatol. To 3 ml of dry pyridine containing 2 g (9.2 mmol) of (±)-sulcatol (Aldrich Chem. Co.) was added 0.7 ml (4.6 mmol) of (1*S*)-(−)-camphanic chloride (Aldrich Chem. Co.). The mixture was stirred at room temperature. After 1 hr, it was cooled in ice-water, and water (50 μl) was added. TLC (ether–light petroleum 1 : 1) showed two products (R_f = 0.65 and 0.80). The mixture was extracted with diethyl ether (8 ml) and CH_2Cl_2 (4 ml), and the organic phase washed successively with saturated aqueous KCl, 1 N HCl, saturated aqueous KCl, and saturated aqueous $NaHCO_3$ (16 ml each). The organic phase was dried with $MgSO_4$. Evaporation of the solvents gave a yellow syrup (1.3 g) that was purified by preparative liquid adsorption chromatography with accelerating gradients (Baeckström, 1996) using Silicagel 60 and mixtures of diethyl ether and light petroleum. The residue obtained was 1.23 g (87%).

The mixture was separated on a Waters Nova-Pak HPLC column (30 cm × 7.8 mm ID) using hexane–2-propanol (500 : 1) at 3 ml/min. The separation was controlled at 203 nm and yielded 90 mg of (−)-1,5-dimethyl-4-hexenyl-(*S*)-(−)-camphanate and 120 mg of (+)-1,5-dimethyl-4-hexenyl-(*S*)-(−)-camphanate from 250 mg of the ester mixture. The esters were identified by: (1) different retention times in the gas chromatograph and identical mass spectra (Figure 1); (2) generation of the respective enantiomeric alcohols by hydrolysis; and (3) pattern of mass fragmentation.

Preparation of (S)-(+)- and (R)-(−)-Sulcatols. To 10 ml methanol containing 120 mg NaOH was added 120 mg of (+)-1,5-dimethyl-4-hexenyl-(*S*)-(−)-camphanate. The mixture was heated under reflux for 1 hr. TLC (diethyl ether–light petroleum 1 : 1) showed complete conversion of the starting material to a single product. Subsequently, water (10 ml) was added and the solution extracted three times with chloroform. The organic phases were dried with $MgSO_4$. Evaporation of the solvent gave 65 mg of (*S*)-(+)-sulcatol (92% yield)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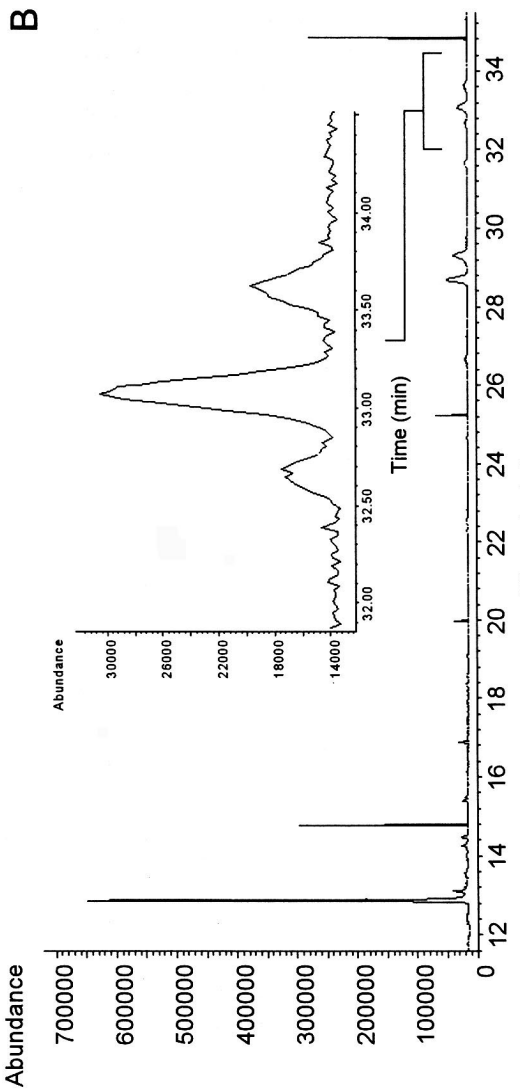

FIG. 1. Total ion current of: (A) (−)- and (+)-1,5-dimethyl-4-hexenyl-(*S*)-(−)-camphanates, including a mass spectrum of the esters, and (B) extract of volatiles released from wheat seedlings with aphids in high density (9 aphids/cm^2) esterified with (1*S*)-(−)-camphanic chloride, including an enlarged area that shows the ratio between diasteroisomeric esters.

with $[\alpha]_J^{20} = +15.3$ (578 nm, c = 0.013, MeOH). Similar treatment of 90 mg of (−)-1,5-dimethyl-4-hexenyl-(*S*)-(−)-camphanate gave 50 mg of (*R*)-(−)-sulcatol (94% yield) with $[\alpha]_J^{20} = -14.1$ (578 nm, c = 0.01, MeOH).

Determination of Enantiomeric Composition of Sulcatol Present in Entrained Mixture of Volatiles. To 500 μl of hexane containing 250 ng of volatile compounds released from wheat infested with a high density of aphids was added 500 μl of hexane containing 100 ng of (1*S*)-(−)-camphanic chloride. The mixture was shaken at room temperature for 3 hr, after which GC-MS did not show the presence of sulcatol. The esters were quantified by GC from calibration curves made with the pure esters. As a control, the reaction of each enantiomer (0.78 mmol) with (1*S*)-(−)-camphanic chloride (0.78 mmol) and 4-dimethylaminopyridine (1 mmol) gave only the respective esters, thus ensuring that the microesterification of the extract would not give collateral products due to acid release.

RESULTS

The enantiomeric mixture of sulcatol was determined as 75% (*R*)-(−)- and 25% (*S*)-(+)-sulcatol (Figure 1). In olfactometric bioassays single enantiomers or a 25% (*R*)-(−)/75% (*S*)-(+)-sulcatol mixture did not show activity towards apterous *R. padi*, while the naturally occurring ratio of (*R*)-(−)- and (*S*)-(+)-enantiomers (75:25) generated a repellency comparable to that obtained when the stimulus was the racemic mixture (Table 1).

TABLE 1. OLFACTOMETRIC RESPONSES OF ONE APTEROUS *R. padi* TO DIFFERENT MIXTURES OF SULCATOL (6-METHYL-5-HEPTEN-1OL) ENANTIOMERS

Stimulus applied	Average time spent in each arm (min)[a]	P[b]
10 ng of (*R*)-(−)-sulcatol	3.45 ± 0.86	0.99
Hexane	3.47 ± 0.59	
10 ng of (*R*)-(−)- and (*S*)-sulcatol (75:25)	2.95 ± 0.45	0.04
Hexane	4.05 ± 0.57	
10 ng of synthetic racemic mixture of sulcatol	2.85 ± 0.35	0.03
Hexane	3.81 ± 0.45	
10 ng of (*R*)-(−)- and (*S*)-(+)-sulcatol (25:75)	2.95 ± 0.78	0.78
Hexane	3.15 ± 0.95	
10 ng of (*S*)-(+)-sulcatol	3.37 ± 0.59	0.80
Hexane	3.27 ± 0.94	

[a] Average ± standard error.
[b] Wilcoxon one tailed rank-sum test for two groups.

DISCUSSION

The synthesis of each enantiomer of sulcatol and the resolution of the racemic mixture were reported earlier. The (*S*)-(+) enantiomer was prepared from L-fucose (Schuler and Slessor, 1977), (*S*)-(−)-lactate (Johnston and Slessor, 1979), (*S*)-(+)-ethyl-3-hydroxybutyrate (Mori, 1981), and by reduction of the respective ketone with biological systems such as bakers's yeast, the anaerobic bacterium, *Clostridium tyrobutyricum*, and the thermophilic anaerobic bacterium, *Thermoanaerobium brockii* (Belan et al., 1987). The (*R*)-(−) enantiomer was prepared chemically from 2-deoxy-D-ribose (Schuler and Slessor, 1977) and biologically by enzymatic reduction of the respective ketone (Belan et al., 1987). Resolution of racemic sulcatol was performed by fractional crystallization of the brucine salt of the phthalic hemiester (Plummer et al., 1976) and by enzymatic resolution (Belan et al., 1987).

In this work, an alternative strategy was employed for obtaining single enantiomers of the alcohol. The racemic mixture was treated with a pure chiral reagent (1*S*)-(−)-camphanic chloride. Subsequently, the esters were separated by preparative HPLC, and finally the enantiomers were generated by basic hydrolysis. Optical rotation values obtained for (*S*)-(+)-sulcatol($[\alpha]_J^{20} = +15.3$) and for (*R*)-(−)-sulcatol ($[\alpha]_J^{20} = -14.1$) compared well with those previously reported by Belan et al. (1987). This classical approach yielded enantiomers with a high optical purity (>99% ee) in a short time (6 hr). Hence, the method proved suitable for the enantiomeric resolution of this secondary alcohol.

The methodology originally designed for the determination of the enantiomeric ratio of sulcatol in our extract of volatiles was based on: (1) synthesis of the enantiomers, (2) enantiomeric analytical separation using a chiral capillary column (α-DEX 120, Supelco), and (3) gas chromatographic analysis of the volatiles using the column mentioned above. However, despite variation of GC parameters such as gas flow, temperature program, and pressure, the column did not separate adequately the racemic mixture of sulcatol. Therefore, the naturally occurring ratio of sulcatol enantiomers was determined in an indirect way. The extract of volatiles, obtained from the system composed of wheat seedling and aphids in high density, was esterified with (1*S*)-(−)-camphanic chloride. Peaks in the GC trace corresponding to the camphanates (same retention time and mass spectra) were quantified. The naturally occurring ratio of enantiomers of sulcatol was determined to be 75(−):25(+).

The olfactometric avoidance response of apterous *R. padi* to racemic sulcatol and to the naturally occurring 75:25 mixture of (*R*)-(−) and (*S*)-(+)-sulcatol [a dose–response curve for this mixture was reported previously by Quiroz et al. (1997)], together with the lack of response to single enantiomers, suggests a synergism between the sulcatol enantiomers. Similar results were described by Borden et al. (1976), who found that *G. sulcatus* only responded to the aggregation pheromone sulcatol when both enantiomers were present and

that the aggregation activity shown by the racemic mixture was greater than the naturally occurring mixture consisting of (35 : 65) of (*R*)-(−)- and (*S*)-(+)-enantiomers.

Although the origin (biological source) of the substance in our system is still unknown, the fact that sulcatol has been reported as a population aggregation pheromone of the beetle *G. sulcatus* (Byrne et al., 1974) and the avoidance behavior shown by apterous *R. padi* towards the naturally occurring mixture of sulcatol suggest that this aphid might use this specific ratio of sulcatol enantiomers as a spacing pheromone.

Earlier reports have shown the low variability of chemical structures used by aphids as pheromones. For instance, the alarm pheromone for several species of aphids has been shown to be (*E*)-β-farnesene (Bowers et al., 1972; Edwards et al., 1973), and the sex pheromone in many species has been shown to comprise the compounds (+)-(4a*S*,7*S*,7a*R*)-nepetalactone and (−)-(1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Dawson et al., 1990; Pickett et al., 1992), species specificity being related to the production of these components in different ratios (Guldemond et al., 1993; Thieme and Dixon, 1996). In that sense, synergism between enantiomers of sulcatol could be a means of achieving species specificity in the spacing behavior of aphids. Studies of the spacing behavior of other aphid species and the compounds responsible for it are underway.

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DIMBOA GLUCOSIDE, A WHEAT CHEMICAL DEFENSE, AFFECTS HOST ACCEPTANCE AND SUITABILITY OF *Sitobion avenae* TO THE CEREAL APHID PARASITOID *Aphidius rhopalosiphi*

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Abstract—The influence of hydroxamic acids (Hx), plant secondary metabolites associated with aphid resistance in wheat, on the host acceptance and suitability of the aphid *Sitobion avenae* to the cereal aphid parasitoid *Aphidius rhopalosiphi* was evaluated. Aphids showed a reduction in mean relative growth rate and in body size in the wheat cultivar with higher Hx level. Reduction in aphid size was related to a decreased success in avoiding parasitoid oviposition. A minor increase in *A. rhopalosiphi* developmental time was observed in aphids feeding on the higher Hx cultivar. Experiments with different concentrations of DIMBOA glucoside, the main Hx in wheat, in artificial diets showed an increase in parasitoid developmental time at the highest concentration, with no change in other performance variables. The evidence is discussed in relation to the compatible utilization of host-plant resistance and biological control in integrated pest management.

Key Words—Tritrophic interaction, aphid, *Sitobion avenae*, parasitoid, *Aphidius rhopalosiphi*, oviposition, defensive behavior, DIMBOA.

INTRODUCTION

Several morphological and/or chemical attributes of a host plant may confer resistance towards herbivorous insects. Resistance to herbivorous insects, however, can also significantly affect the performance of their natural enemies (Price et al., 1980; Vet and Dicke, 1992; Hare, 1992) producing negative, compatible,

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or even synergistic interactions between host-plant resistance and biological control.

In particular for aphids, plant allelochemicals such as volatile, epidermal, and internal metabolites constitute potential resistance factors that can play important roles in aphid–plant interactions (Niemeyer, 1990; Pickett et al., 1992). In relation to tritrophic effects, van Emden and Wratten (1990) reviewed the influence of the plant on aphids and their natural enemies, suggesting that the emphasis on deleterious tritrophic effects might not reflect the prevalent situation.

In wheat and other Poaceae, hydroxamic acids (Hx), a family of plant secondary metabolites, have been identified as resistance factors showing deterrent and antibiotic properties against cereal aphids (Niemeyer and Pérez, 1995). These compounds could be a target for breeding programs aimed at increasing resistance towards cereal aphids (Escobar and Niemeyer, 1993). Nevertheless, in integrated pest management strategies it is necessary to evaluate the potential influence of the proposed increase in Hx levels on biological control agents.

With regard to the process of host selection in parasitoids of cereal aphids, host acceptance and suitability might be influenced by plant secondary metabolites such as Hx. Studies addressing host acceptance of cereal aphid parasitoids in relation to these metabolites are not available. However, in relation to aphid suitability Fuentes-Contreras et al. (1996) found a small tritrophic effect, ca. 5% increase in developmental time, on the cereal aphid parasitoid *Aphidius rhopalosiphi* De Steph. feeding on wheat (*Triticum aestivum* L.) when compared to those feeding on oat (*Avena sativa* L.), a cereal lacking Hx.

In order to evaluate further the potential influence of Hx on parasitoids of cereal aphids, we studied the effect of wheat cultivars with different Hx levels on the host acceptance behavior of the cereal aphid parasitoid. *A. rhopalosiphi* and the respective defensive reactions to parasitoid attack of the English grain aphid *Sitobion avenae* (F.). In addition, we evaluated the influence of 2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-glc), the main Hx in wheat, on *S. avenae* suitability for *A. rhopalosiphi* development when aphids fed on wheat cultivars or artificial diets with different levels of Hx.

METHODS AND MATERIALS

Plant Material and Aphid and Parasitoid Stock Culture. All experiments were performed with *S. avenae* from a stock culture maintained on oat (cv. Nehuén) and *A. rhopalosiphi* from a stock culture maintained on *S. avenae* reared on the same oat cultivar. The experiments involving plants were performed using two spring wheat cultivars with different levels of DIMBOA-glc: Huenufén ($\bar{X} = 1.72 \pm 0.12$ mmol/kg fresh wt) and Naofén ($\bar{X} = 3.02 \pm 0.17$

mmol/kg fresh wt) (concentration on primary leaf of 6-day-old seedlings, $N = 6$).

Plant Analysis of Hydroxamic Acids and Their Isolation. Quantification of DIMBOA-glc in plants was performed with reverse-phase high-performance liquid chromatography (HPLC), as previously described by Weibull and Niemeyer (1995). DIMBOA-glc for HPLC standards and experiments with artificial diets was isolated from *Zea mays* L. (CV T55s) according to the protocol described by Hartenstein et al. (1993). The identity of the products obtained was checked by HPLC against standards provided by Dr. Dieter Sicker (Leipzig University, Leipzig, Germany). Purity was also evaluated by HPLC and ranged between 95 and 98%.

Influence of Wheat Cultivars on Host Acceptance by Parasitoid and Aphid Defensive Behavior. Before the beginning of the trials, colonies of *S. avenae* were transferred for at least four generations to the above-mentioned wheat cultivars (Huenufén and Naofén). Two synchronized third-instar nymphs, one from each wheat cultivar, were placed singly on oat leaves inside petri dishes (35 mm diameter, 10 mm height). Since volatile compounds of the plant might affect attack rate of the parasitoid (Powell and Wright, 1992; Braimah and van Emden, 1994), only oat leaves were used in the experimental arena, so wheat volatiles from different cultivars that would affect parasitoid behavior during the experiments were absent. After allowing aphids to settle for 10 min, a naive female of the parasitoid was introduced in the arena. All parasitoid females were mated, 2–3 days old, and were used only once. The behavior of the parasitoid and the aphid was observed under a Nikon stereoscopic microscope and recorded continuously with the software "The Observer." The observation period lasted for 5 min. The following parasitoid and aphid behavioral events were recorded: (1) encounter—female parasitoid approaching the aphid and tapping the aphid body with its antennae; (2) attack—female parasitoid bending the abdomen forward and reaching or not reaching the aphid body; (3) stab—female parasitoid reaching the aphid body with the ovipositor; (4) kicking—aphid kicking backwards with its hind legs after being contacted by the foraging parasitoid; and finally (5) cornicle secretion—droplets of cornicle secretion observed after a parasitoid contact.

Influence of Wheat Cultivars on Parasitoid and Aphid Performance. Mean relative growth rate (MRGR) of *S. avenae* was evaluated during a four-day period. Synchronized second-instar aphids were placed inside clip-cages on the wheat seedlings as described previously in Fuentes-Contreras et al. (1996). All experiments were performed with 6-day-old, growth stage 11 (Zadoks et al., 1974) seedlings grown under the following environmental conditions: 25 ± 1°C, 16L:8D, 3000 lux. In the same way, the effects of the wheat cultivars on *A. rhopalosiphi* development were evaluated following the protocol described in Fuentes-Contreras et al. (1996).

Artificial Diet Experiments. Artificial diets were prepared following Febvay et al. (1988). Synchronized third-instar aphids from the stock culture were parasitized as described above for plants, but the aphids were transferred to Plexiglas cages with artificial diet inside Parafilm sachets provided instead of wheat seedlings. Two DIMBOA-glc concentrations (2 and 4 mM) and a control without the compound were used in the experiments. These concentrations represent physiological concentrations found by the aphid while feeding in the cultivars used in our experiments (Givovich and Niemeyer, 1995).

RESULTS

Effect of Wheat Cultivars on Aphid–Parasitoid Interaction. Behavior of the female parasitoid in terms of frequency of encounters and attack attempts on the host was not significantly different between the two wheat cultivars. Additionally, aphid defensive reactions, such as kicking or production of cornicle secretion, were not significantly different between wheat cultivars (Table 1). However, the frequency of stabs was significantly higher on cultivar Naofén, which shows a higher level of Hx than the cultivar Huenufén (Table 1).

Effect of Wheat Cultivars in Aphid and Parasitoid Development. MRGR of the aphid was significantly lower in cultivar Naofén (Table 2). Since the sex ratio of the parasitoids was not significantly different between wheat cultivars (Table 2), data from other variables of parasitoid performance were pooled for both sexes. Total developmental time of the parasitoids significantly increased by approximately one day in cultivar Naofén (Table 2). This increase in developmental time was accounted for by a significant increase in egg–larval developmental time, whereas pupal developmental time remained not significantly different between cultivars (Table 2). No other variables, such as survival or body mass, were affected by the wheat cultivars.

Aphids and Parasitoid Performance in Artificial Diets. MRGR of the aphid significantly decreased as DIMBOA-glc concentration increased (Table 3). In the same way as for the experiment with plants, data from both parasitoid sexes were pooled, based on nonsignificant differences in sex ratio between treatments. A significant increase in total developmental time of the parasitoids was observed in DIMBOA-glc diets in relation to the control diet, although there was no significant difference between 2 and 4 mM DIMBOA-glc (Table 3). The increase in total developmental time was associated with a significant increase in the egg-larval developmental time of treatments containing DIMBOA-glc. There were no significant differences in pupal developmental time (Table 3). In addition, overall parasitoid survival was not significantly affected by DIMBOA-glc, but a partial reduction in egg–larval survival was detected as DIMBOA-glc concentration increased (Table 3). No further effects were detected in other variables of parasitoid performance.

TABLE 1. BEHAVIORAL EVENTS DURING *S. avenae* ACCEPTANCE PROCESS BY *A. rhopalosiphi*[a]

Wheat cultivar Hx level (mean ± SE, mmol/kg fresh wt)	*A. rhopalosiphi*			*S. avenae*		
	Encounter	Attack	Stab	Kick	Cornicle secretion	Body size mg
Huenufén (1.72 ± 0.12)	2.95(0, 34)a	0.91(0, 19)a	0.23(0.06)a	2.50(0.35)a	0.18(0.11)a	0.230(0.009)a
Naofén (3.02 ± 0.17)	2.45(0, 36)a	1.05(0, 14)a	(0.48(0.06)b	2.06(0.27)a	0.23(0.12)a	0.124(0.008)b

[a]Values (number of events) given are means; standard errors in parenthesis. Values in each column followed by the same letter are not significantly different according to the Wilcoxon matched-pair test ($\alpha = 0.05$). $N = 22$.

TABLE 2. EFFECT OF WHEAT CULTIVARS ON PERFORMANCE OF *S. avenae* AND *A. rhopalosiphi*[a]

Wheat cultivars Hx level (mean ± SE, mmol/kg fresh wt)	*S. avenae* MRGR (μg/μg/day)	*A. rhopalosiphi* development time (days)			*A. rhopalosiphi* survival (%)	*A. rhopalosiphi* body mass (mg)	*A. rhopalosiphi* secondary sex ratio (F/M)
		Total	Egg-larval	Pupal			
Huenufén (1.72 ± 0.12)	0.330a	8.95a	5.41a	3.55a	100a	0.0399a	0.48a
Naofén (3.02 ± 0.17)	0.262b	10.23b	6.64b	3.59a	100a	0.0363a	0.60a

[a] Values given are means. Values in the same column followed by the same letter are not significantly different according to the Kruskal-Wallis test (α = 0.05). Sample size: *S. avenae*, N = 21; *A. rhopalosiphi*, N = 25.

TABLE 3. EFFECT OF DIMBOA-glc IN ARTIFICIAL DIETS ON PERFORMANCE OF *S. avenae* AND *A. rhopalosiphi*[a]

DIMBOA-glc concentration	*S. avenae* MRGR (μg/μg/day)	*A. rhopalosiphi* development time (days)			*A. rhopalosiphi* survival (%)		*A. rhopalosiphi* body mass (mg)	*A. rhopalosiphi* secondary sex ratio (F/M)
		Total	Egg-larval	Pupal	Total	Egg-larval		
0 mM	0.194a	16.33a	9.01a	7.40a	65a	80a	0.0328a	0.58a
2 mM	0.128b	16.72ab	9.79ab	7.28a	60a	65b	0.0332a	0.5a
4 mM	0.105c	17.8b	10.46b	7.25a	50a	55b	0.0316a	0.55a

[a] Values given are means. Values in each column followed by the same letter are not significantly different according to the Kruskal-Wallis test ($P \leq 0.05$). Sample size, *S. avenae*, $N = 21$, and *A. rhopalosiphi*, $N = 40$.

DISCUSSION

Several studies have shown that different cereal species (Reed et al., 1992; Messina et al., 1995; Fuentes-Contreras et al., 1996) and cultivars (Kuo, 1984) may influence host suitability to natural enemies of aphids. Since Hx in wheat and other Poaceae exert a deleterious effect on aphid performance (Niemeyer and Pérez, 1995), they represent a potential mechanism to explain tritrophic effects involving natural enemies (e.g., Martos et al., 1992). In the present study, this negative effect on aphids was expressed in the reduction of MRGR, and consequently of body size, on the wheat cultivar with the higher Hx level. Aphid body size influences the success of aphid defensive reactions against attack by natural enemies (Gerling et al., 1990, Kouamé and Mackauer, 1991; Gross, 1993). Our results showed that aphids grown on wheat cultivar Naofén (high Hx level) were found and attacked by parasitoids at freqencies similar to aphids from the Huenufén cultivar (low Hx level). Likewise, aphids from both cultivars showed kicking and production of cornicle secretion with similar frequencies. However, the stabbing success of the parasitoids was lower in aphids from the susceptible cultivar Huenufén. This result may be tentatively explained by an increase in the success of avoidance of parasitoid stabbing in each kicking reaction of bigger aphids from the susceptible cultivar Huenufén, i.e., larger aphids would kick more effectively. Furthermore, these results are comparable to those of Campos et al. (1990), who also detected an increase in parasitization by *Diadegma terebrans* (Gravenhorst) on larvae of the European corn-borer reared in artificial diets containing Hx with respect to control diets (Campos et al., 1990).

An increase in total developmental time accounted for by an increase in egg–larval developmental time was observed in the high-Hx cultivar Naofén and in the artificial diet with highest Hx concentration. Concentrations of secondary metabolites in artificial diets should reflect physiological concentrations experienced by the aphid when feeding from sieve elements. Based on data from Givovich et al. (1994), it was possible to estimate that DIMBOA-glc concentrations in the phloem sap of the cultivars used here range from 0 to 4 mM. Since the range of DIMBOA-glc concentrations provided in the present artificial diets lies within the range mentioned above, results of the experiments with artificial diets substantiate those obtained with plants.

Aphid ingestion volumes are much lower from diets than from phloem sap (Klingauf, 1987). Thus, concentrations used in the artificial diets were probably exposing the parasitoids developing inside the aphids to rather low levels of Hx. However, higher Hx concentrations in diets produce antifeeding effects on aphids (Niemeyer et al., 1989) and hence preclude proper testing of the effect of the compound on parasitoid performance. Furthermore, higher concentrations of Hx in diets reduce aphid survival (Niemeyer and Peréz, 1995) and consequently

would increase parasitoid mortality within the aphids. This effect could be responsible for the reduction in parasitoid survival during its egg–larval development. Development time of the parasitoid was reduced in comparable magnitude by DIMBOA-glc concentration in plants and in artificial diets (ca. 1 day). However, total development time of the parasitoid was much lower in the plants than in the diets, and hence in relative terms, the increase in development time in the plant is ca. 10%, while in the diets it is ca. 5%. This difference could be ascribed to the lower ingestion of the compound in diets.

In conclusion, the tritrophic effect of DIMBOA-glc on *A. rhopalosiphi* is confined to behavior, as an increase in successful stabs in aphids reared on wheat cultivars that have higher levels of the compound, and to life history traits, as a minor increase in development time. From a practical point of view, our results support the compatibility of biological control with an increase in Hx levels through breeding programs, as suggested by Campos et al. (1990) and Martos et al. (1992). Plants with higher Hx concentration would reduce aphid MRGR, thus increasing the proportion of smaller aphids in the population and facilitating parasitoid oviposition attacks. Moreover, the deleterious influence of Hx on overall survival of the parasitoid is not significant, and any potential reduction in parasitoid body size seems to be compensated by increased development time.

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LEARNED AVERSION TOWARDS OXALIC ACID-CONTAINING FOODS BY GOATS: DOES RUMEN ADAPTATION TO OXALIC ACID INFLUENCE DIET CHOICE?

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Abstract—The ability of goats to learn to avoid flavored alfalfa hay enriched with oxalic acid was investigated in an indoor choice experiment. In addition, the influence of rumen adaptation to oxalic acid on the strength of avoidance behavior was studied. The experiment consisted of four nine-day conditioning periods during which 24 goats were fed hay with or without added oxalic acid for two-day bouts in sequence. Sensory differences between the hays were emphasized by incorporating artificial flavors (fenugreek vs. apple). Half the goats were adapted to oxalic acid by daily oral administration of 0.6 mmol/kg live wt/day of free oxalic acid, which successfully generated an active oxalic acid-degrading rumen microbial population. At the end of each conditioning period, short-term preference for the hay diets was measured in 20-min choice trials. Plasma calcium concentrations were measured during each conditioning period as an indicator of the physiological effect of oxalic acid ingestion. Animals showed a chronic mild hypocalcemia (initial baseline values were 115 mg Ca/dl plasma vs. 89 mg Ca/dl plasma for the mean of the rest of the samplings during the conditioning phase). Preference for the oxalic acid-containing and the control diets was similar prior to conditioning. During conditioning, preference for the oxalic acid-containing diet was diminished (e.g., preference ratios during third conditioning period averaged 0.30 for oxalic acid-containing diets and 0.70 for control diets; $P < 0.05$). In preference tests carried out one, three, and five weeks after the last conditioning period, preference progressively returned to preconditioning levels. The rumen

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adaptation treatment did not result in different behavioral responses to the oxalic acid-containing foods. In this experiment goats learned to avoid a mildly aversive agent present in their food when given the opportunity to associate the sensory properties of the food with its physiological effects. Prior physiological adaptation to the aversive compound did not influence the behavioral response.

Key Words—Conditioned aversions, diet selection, physiological adaptation, ruminants, secondary compounds.

INTRODUCTION

Ruminants select a diet from a wide array of plant species. They generally select foods that meet their nutritional needs and avoid foods that cause harmful effects (Provenza, 1995). Their diet choice may involve a component of trial-and-error learning based on sampling foods and monitoring the nutritional and physiological consequences of their ingestion. Changes in the intake or selection of food items may depend on the degree to which postingestive consequences are negative or positive (du Toit et al., 1991). Animals are likely to require cues in order to be able to determine which foods are associated with which postingestive effects. For example, they can form associations between the flavor of a food and its negative postingestive consequences (Launchbaugh et al., 1993) and these associations can lead to conditioned food aversions (CFA) (Pfister et al., 1992; Launchbaugh and Provenza, 1994; Provenza et al., 1994). The strength and persistance of a conditioned food aversion has been shown to be closely related to the intensity of the detrimental physiological effects of that food (Ralphs, 1992).

Many plants contain toxins that appear to act as deterrents to feeding by mammalian herbivores (Bryant et al., 1992). Adaptation of herbivores to potentially toxic plant secondary compounds found in their diets may influence their food choice (Provenza et al., 1992). If this were the case, ruminants would be expected to sample small amounts of novel, potentially toxic plants, but following continued ingestion, the induction of detoxification pathways could enable them to increase their utilization of these plants without detrimental effects. Ruminal degradation is the primary defense against some dietary toxins, for example oxalates (Allison and Reddy, 1984). Oxalic acid and its salts, consumed in sufficient quantity, can cause renal toxicity and hypocalcemia (James, 1972; Libert and Franceschi, 1987, Von Burg, 1994). A previous experiment with sheep and goats showed that administration of free oxalic acid led to changes in the rate of oxalic acic breakdown in the rumen, particularly of goats, thus protecting the host animal from toxic symptoms (Duncan et al., 1997).

Two hypotheses were tested in the experiment reported here: the first was that ruminants are able to form associations between the flavors of foods con-

taining natural detrimental compounds and their physiological effects and subsequently learn to avoid them. The second was that the strength of avoidance is modulated by the capacity of herbivores to minimize physiological effects of harmful plant components via physiological adaptation.

METHODS AND MATERIALS

Preliminary Acceptability Tests

Prior to the main experiment, acceptability trials were conducted to allow appropriate selection of flavors for use in the main experiment. For this purpose, flavored hays were prepared with four flavors (fenugreek, orange, aniseed and apple; International Additives Ltd., Wallasey, UK; level of inclusion 7.5 g/kg). Each possible pair of flavored hays was offered to six adult female goats (different from the animals used for the main experiment) and the weight of each hay consumed was recorded. The conduct of the acceptability trials was identical to preference tests conducted during the main experiment (see below). These trials showed that fenugreek and apple produced the most extreme and consistent choices, with fenugreek being preferred over apple when these two flavored hays were offered concurrently.

Experimental Animals and Diets

Twenty-four castrated Scottish Cashmere goats (mean live weight 37.5 kg, SE 1.10) were used. All animals were offered a background diet of dried grass pellets [metabolizable energy (ME) content = 10 MJ/kg dry matter; crude protein (CP) content = 156 g/kg dry matter; neutral detergent fiber (NDF) content = 497 g/kg dry matter) at 1.2 times their estimated maintenance requirements for energy for two weeks prior to commencing the experiment.

The experimental diets were formulated with chopped, molassed alfalfa hay (trade name HiFi; estimated ME content = 71.1 MJ/kg dry matter, CP content = 117 g/kg dry matter; Dengie Farm Feeds, Essex), as follows: Pair 1 consisted of APcont—Alfalfa hay with the avoided flavor (apple) incorporated, and FEoxal—alfalfa hay with the preferred flavor (fenugreek) incorporated + 60 mmol of oxalic acid/kg hay.

Pair 2 consisted of FEcont—alfalfa hay with the preferred flavor (fenugreek) incorporated, and APoxal—alfalfa hay with the avoided flavor (apple) incorporated + 60 mmol of oxalic acid/kg hay.

The flavors (level of inclusion 7.5 g/kg hay) as well as the oxalic acid were first mixed with maize starch (dilution 1:4) and subsequently thoroughly mixed with the alfalfa hay in a cement mixer for 5 min.

Experimental Design

Conditioning Periods. The experiment consisted of four consecutive conditioning periods lasting nine days each, during which the animals were offered each member of one of the experimental diet pairs in sequence, interspersed with rest periods during which the background diet was offered (Table 1). In this way, different flavors were associated with either the oxalic acid or the control diet. The rest periods were designed to minimize any residual short-term toxic effects from the previous diet. Within conditioning periods, experimental diets were offered in two-day bouts with one diet being offered exclusively on days 1 and 2 and the other exclusively on days 5 and 6. Each experimental diet pair was offered to one group of animals (12 goats) with the order of offering the diets being reversed in half of the goats, but remaining consistent within animals (Table 1). The experimental diets were offered as the sole source of food at 1.2 times estimated daily maintenance ME requirements split into two equal meals (at 09:30 hr and at 15:00 hr). On days when experimental diets were not offered, the background diet was also offered at 1.2 times maintenance ME requirements.

Adaptation Treatment. Oxalic acid (Aldrich, Dorset, UK) contained in gelatin capsules (Davcaps, Monmouth, UK) was administered to half of the animals (adapted) by once daily oral dosing (at 08:00 hr) of 0.6 mmol of oxalic acid/kg live weight/day, and the remaining animals (unadapted) received empty capsules. The level of administration was based on a previous experiment (Duncan et al., 1997) where the dose level was shown to prime the rumen microflora to oxalic acid ingestion, without significant toxic effects. During one preliminary adaptation week, the dose was increased from zero to 100% of the full dose in equal stages over five days and then the full dose given for two more days to ensure adaptation. A period of one week was found in the previous experiment to be of sufficient duration to allow adaptation of the rumen microbial population (Duncan et al., 1997). Oxalic acid was then administered daily to the "adapted" animals at the full dose rate, except on days during which they received the experimental diet containing oxalic acid, when the dose was withheld. This was to avoid overwhelming the rumen microbial population, which would otherwise have been exposed to oxalic acid from both the diet and the priming dose. The dose was not withheld from equivalent animals consuming the oxalic acid-free diet since this could have resulted in microbial death and loss of oxalic acid-degrading ability. The adaptation treatment was designed to maintain rumen microbes in an adapted state but not to lead to postabsorptive differences between adapted and nonadapted animals. This was achieved by keeping the daily rate of artificial administration low in contrast to the larger and more abrupt exposure from the experimental diets. Unadapted animals received empty gelatin capsules daily throughout the experiment.

TABLE 1. TIMETABLE OF ORDER OF OFFERING EXPERIMENTAL DIETS DURING ONE CONDITIONING PERIOD

		Fenugreek associated with oxalic acid				Apple associated with oxalic acid			
		Adapted goats		Unadapted goats		Adapted goats		Adapted goats	
		$N = 3$	$N = 3$	$N = 3$	$N = 3$	$N = 3$	$N = 3$	$N = 3$	$N = 3$
Day 1	Experimental diet	FEoxal[a]	APcont[b]	FEoxal	APcont	FEcont[c]	APoxal[d]	FEcont	APoxal
Day 2	Experimental diet	FEoxal	APcont	FEoxal	APcont	FEcont	APoxal	FEcont	APoxal
Day 3	rest								
Day 4	rest								
Day 5	Experimental diet	APcont	FEoxal	APcont	FEoxal	APoxal	FEcont	APoxal	FEcont
Day 6	Experimental diet	APcont	FEoxal	APcont	FEoxal	APoxal	FEcont	APoxal	FEcont
Day 7	rest								
Day 8	rest								
Day 9	Preference test	FEoxal/ APcont	FEoxal/ APcont	FEoxal/ APcont	FEoxal/ APcont	APoxal/ FEcont	APoxal/ FEcont	APoxal/ FEcont	APoxal/ FEcont

[a] Alfalfa hay with fenugreek flavor incorporated (7.5 g/kg) + 60 mmol of oxalic acid/kg.
[b] Alfalfa hay with apple flavor incorporated (7.5 g/kg).
[c] Alfalfa hay with fenugreek flavor incorporated (7.5 g/kg).
[d] Alfalfa hay with apple flavor incorporated (7.5 g/kg) + 60 mmol of oxalic acid/kg.

Conduct of Preference Tests. A preference test was carried out just prior to the first conditioning period and at the end of each of the four conditioning periods (day 9, Table 1). All animals were given a choice between equal amounts of the two different diets that they had been receiving during conditioning. The tests lasted for 20 min, and the goats were offered 1.2 times daily maintenance ME requirements of each of the two different diets. The high amounts of food offered were considered necessary to avoid the depletion of either diet influencing preference. Consumption was determined by weighing the remains of each diet, and the results were calculated as the amount of one diet consumed, expressed as a proportion of total intake, during the 20-min trial (defined as preference ratio). Three further preference tests were performed one, three, and five weeks after the last conditioning period to test for the persistance of any conditioned food aversion in the absence of continual reinforcement. The diets offered during the preference tests conducted during this persistence period were exactly the same as those used for conditioning.

In the description of the results, the first test is referred to as the Pre test, the tests performed at the end of each conditioning period the During tests and the tests carried out one, three, and five weeks after the last conditioning period the Post tests.

Sample Collection. In order to measure oxalic acid degradation rate, rumen samples (20 ml) were collected, under anaerobic conditions, by stomach tube at 09:00 hr on days 3 and 9 of each conditioning period and just prior to starting the adaptation week. Rumen samples were also taken at 09:00 hr on the day of each preference test during the persistence period.

Blood samples (10 ml) were collected from the jugular vein into heparinized evacuated tubes (Vacutainer, Becton Dickinson, Oxford, UK) to measure plasma calcium concentrations as an indicator of oxalic acid toxicity. These samples were taken just prior to the start of the adaptation period and on days 2 and 6 of each conditioning period, 1 hr after the afternoon administration of the diets.

Measurements and Analyses

Samples of ruminal contents were kept at 37°C and processed within 60 min of collection. Rates of oxalic acid degradation were measured by adding ^{14}C-labeled oxalic acid to rumen fluid in vitro and capturing evolved $^{14}CO_2$, by a modification of the method of Allison et al. (1977). The details of the method are described by Duncan et al. (1997).

Plasma calcium concentrations were determined by automated flame microsampling (Voth, 1981).

Statistical Analysis

Split-plot analysis of variance was used to examine the effect of diet, adaptation treatment, order of offering diets and period on preference ratios for

fenugreek-associated diets, rate of oxalic acid degradation in the rumen, and plasma Ca concentrations. Variance associated with diets, adaptation treatment, and order of offering the diets was estimated between animals. Variance associated with period and interactions with period was estimated within animals. Polynomial contrasts were used to provide a closer examination of the effects of period and period × treatment interactions. The residual mean square was split into components for each contrast because there was evidence that these components differed between contrasts. This also ensured that the analysis was not influenced by the effects of correlation among these repeated-measures data.

For oxalic acid degradation data, analysis of variance was used to test the effect of the experimental diets on rate of oxalic acid degradation in the rumen. To this end, the effect of diet (with or without oxalic acid) and time (immediately before or after offering the diet) on degradation rate was tested.

Rates of degradation of oxalic acid and plasma Ca concentrations were log-transformed and preference ratios were subjected to angular transformation to improve the homogeneity of the variance. Data were analyzed with Genstat 5 (Lawes Agricultural Trust, 1989).

RESULTS

Preference Tests. Neither the effect of the adaptation treatment nor the order of offering diets affected preference ratios in preference tests conducted during the conditioning periods or during the subsequent persistence phase.

Differences between the preference ratios for the two fenugreek-flavored diets (FEoxal and FEcont) showed a significant quadratic trend ($P < 0.01$) with differences between the preference ratios of the two diets increasing during the conditioning phase, and then decreasing in the postconditioning, persistence phase of the experiment (Figure 1). Preference ratios thus diverged during the conditioning phase and then converged following that phase, indicating the development of aversions during the conditioning phase towards the diets associated with the oxalic acid. Preference ratios in tests conducted at the end of each of the four conditioning periods were significantly lower than the mean of values before and after the conditioning phase (During vs. Pre + Post contrasts, $P < 0.05$). There was no evidence for persistence of developed aversions after the conditioning had stopped (Pre vs. Post contrast; $P > 0.05$). There were no significant interactions.

The total intake of the experimental diets over the 20-min preference tests was lower during the preconditioning test than during the conditioning and persistence phases (444 vs. 495 and 508 g, respectively; Pre vs. During + Post contrast; $P < 0.01$). Absolute hay intake during preference tests increased during the conditioning phase (linear contrast within During phase, $P < 0.05$) and was similar among the three preference tests carried out one, three, and five

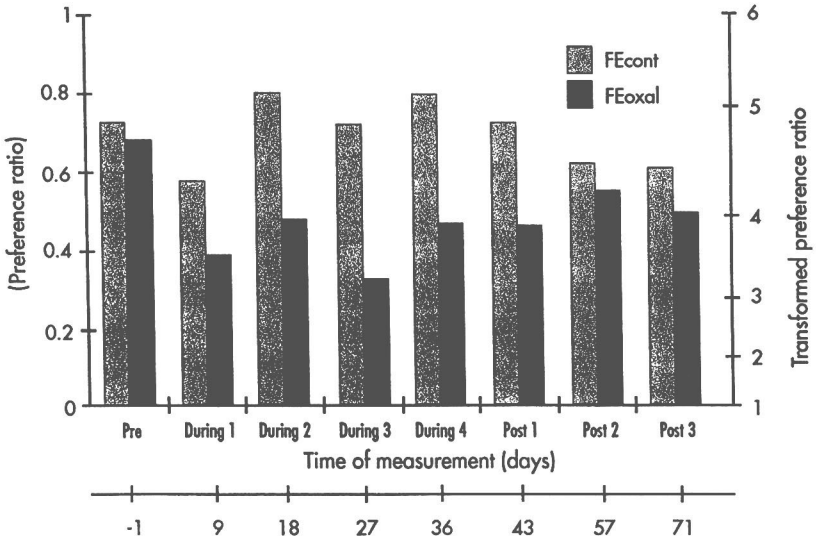

FIG. 1. Preference ratios for the fenugreek-flavored diets (FEcont and FEoxal) for the Pre preference test (control) and for tests performed at the end of each conditioning period (During-1, During-2, During-3, and During-4) and one, three and five weeks after the last conditioning period (Post-1, Post-2, and Post-3, respectively). The *y* axis on the right of the histogram presents the transformed scale. SED (standard error of difference) for the right-hand *y* axis, for the interaction between diet and period = 0.635.

weeks after the last conditioning period (linear contrast within Post phase; $P > 0.05$). There was no effect of either the diet pair received, the adaptation to oxalic acid, or the order of offering diets on absolute hay intake by goats during the preference tests.

Physiological Variables. Daily oral dosing of oxalic acid caused an increase in the rate of degradation of oxalic acid in the rumen in adapted animals ($P < 0.001$; Table 2). Average rates of oxalic acid degradation were 0.360 μmol/ml rumen fluid/hr for adapted animals and 0.198 μmol/ml rumen fluid/hr for non-adapted animals. Rates of degradation, expressed as means of values measured on days 3 and 9 for the conditioning periods, increased throughout the experimental period ($P < 0.001$). Rates of degradation of oxalic acid measured throughout the conditioning phase in unadapted goats were significantly lower before than after having received the diet associated with oxalic acid (APoxal or FEoxal), showing average values of 0.152 vs. 0.240 μmol/ml rumen fluid/hr, respectively [diet (control vs. oxalic acid associated) × time (before vs. after) interaction, $P < 0.001$].

TABLE 2. RATES OF OXALIC ACID DEGRADATION IN RUMEN AND PLASMA CALCIUM CONCENTRATIONS IN ADAPTED AND UNADAPTED GOATS PRIOR TO ADAPTATION WEEK (INITIAL BASELINE), DURING CONDITIONING PERIODS AND AFTER LAST CONDITIONING PERIOD

	Initial baseline	Conditioning period				After conditioning (weeks)				
		During-1	During-2	During-3	During-4	Post-1	Post-2	Post-3	SED^{1a}	SED^{2a}
Rate of rumen oxalic acid degradation (μmol/nl rumen fluid/hr) (transformed values in parenthesis)										
Adapted	0.101	0.336	0.398	0.477	0.412	0.515	0.645	0.527		
	(−2.287)	(−1.091)	(−0.920)	(−0.741)	(−0.886)	(−0.662)	(−0.438)	(−0.640)		
									(0.2114)	(0.1749)
Unadapted	0.150	0.163	0.133	0.200	0.272	0.242	0.237	0.316		
	(−1.897)	(−1.811)	(−2.016)	(−1.610)	(−1.301)	(−1.416)	(−1.438)	(−1.153)		
Plasma calcium concentration (mg/dl) (transformed values in parenthesis)										
Adapted	119.41	92.24	91.43	87.33	84.31					
	(4.755)	(4.522)	(4.510)	(4.459)	(4.424)					
									(0.0453)	(0.0432)
Unadapted	110.75	93.65	91.67	86.09	87.68					
	(4.672)	(4.537)	(4.517)	(4.453)	(4.473)					

[a] SED^1 denotes standard error of difference for the interaction between adaptation treatment and period for between-animal comparisons, SED^2 is for within-animal comparisons.

Calcium concentrations in plasma (Table 2) were not significantly affected by adaptation to oxalic acid. There was, however, an effect of the period, with a significant decrease between the initial baseline sampling and the rest of the samplings carried out during the conditioning phase (Pre vs. During contrast, $P < 0.01$). Furthermore, calcium concentrations of plasma samples taken immediately after the goats had received the hay with oxalic acid incorporated (APoxal and FEoxal) were significantly lower than calcium concentrations of plasma samples taken immediately after the goats had received the control diet (FEcont or APcont), showing average values of 88.7 vs. 89.72 mg/dl ($P < 0.05$).

DISCUSSION

The objectives of the experiment were to determine whether goats could learn to avoid a mildly detrimental natural secondary compound present in their diet and whether the strength of the aversion developed was modified by physiological adaptation of the animal to the secondary compound.

By offering differently flavored diets, the aim was to give the animals the opportunity to associate the different flavors with the physiological consequences of consuming diets with or without added oxalic acid. It is possible that the flavor of the oxalic acid itself might have been sufficient to provide goats with the sensory cues necessary to distinguish the two diets. Two flavors not equally preferred by goats were added to the feed, however, to reinforce sensory differences between the diets. In pair 1, the preferred flavor (fenugreek) was associated with the oxalic acid and the rejected one (apple) used as the contrast. The reverse association (fenugreek without oxalic acid vs. apple with oxalic acid) was included as pair 2 to ensure that any changing preferences observed during the experiment were related to oxalic acid inclusion and not to temporal changes in acceptability for the flavors. This maximized the animals' opportunity to identify the diets and hence express any conditioned food aversion that might develop.

The different physical characteristics of the background and experimental diets ensured that potentially subtle differences between the experimental diets were not confused with similar differences between the experimental and background diets.

The goats learned to avoid the food containing oxalic acid after they had been given the opportunity to associate its sensory attributes with its physiological effects. It has been widely reported that ruminants sample novel foods and, depending on the resultant postingestive consequences (i.e., whether or not they experience malaise), either increase intake of those foods or avoid them and form so-called conditioned food aversiosn (CFA) (Zahorik et al., 1990; Provenza et al., 1990; 1992; Pfister et al., 1992; Launchbaugh and Provenza,

1994). In this experiment, goats initially selected the fenugreek-flavored food (even in pair 1, where it was associated with oxalic acid) because they were hedonically attracted to that flavor. However, after being offered both experimental diets during the conditioning periods and experiencing the postingestive consequences of flavors associated with oxalic acid, their preference for the food containing the secondary compound was diminished and they selected a higher proportion of the alternative food, regardless of the flavor associated with it. The fact that the animals only developed a mild aversion was probably due to the mild postingestive consequences caused by the food containing the oxalic acid, since previous studies have demonstrated a close relationship between the degree of aversion and the strength of the negative consequences (du Toit et al., 1991; Provenza, 1995). The amount of oxalic acid ingested may have set a limit to the amount of food goats chose to ingest through a toxin satiation mechanism (see Wang and Provenza, 1997).

Once the goats had acquired the aversion towards the diet containing the oxalic acid, there was no evidence that they retained, in absence of continuous reinforcement, the memory of its negative consequences. Although CFAs have been reported to persist in some cases for 60 days without exposure (Thorhallsdottir et al., 1987), persistence of mild aversions to emetic drugs does not generally last for long (Ralphs, 1992). The current results extend the findings of previous work using artificial emetic drugs and demonstrate that a mild aversion to a natural toxic stimulus is extinguished relatively rapidly in the absence of on-going experience of physiological consequences. The adaptive value of flexibility of learned aversions is clear when one considers the temporal and spatial variability in secondary compound concentrations commonly observed (Coley et al., 1985). Herbivores probably acquire short-term aversions to foods repeatedly during their lives (Provenza, 1995; Burritt and Provenza, 1996) as a consequence of their sampling behavior. Obviously, the long-term persistence of aversions plays an important role when the food is very toxic. However, when the food merely causes mild detrimental effects, aversion to its sensory properties (for example, its flavor) would be beneficial if retained for only short periods. This allows relearning appropriate to changes in the concentrations of toxins in plants associated with plant phenology and environmental factors and changes in the physiological consequences as animals adapt to the secondary compounds in their diets (du Toit et al., 1991; Launchbaugh et al., 1993; Forbes and Kyriazakis, 1995).

The absolute intake of the experimental diets consumed during the preference tests increased during the experiment, and this may have been caused by the initial novelty of the food. Ruminants generally sample novel foods, which ensures that they do not overingest toxic plants (Provenza et al., 1994, 1995). The goats did not completely stop eating the food containing oxalic acid, indicating that they may be able to adjust their intake of detrimental foods so that

ingestion of the toxin is kept to a tolerable level (du Toit et al., 1991). Animals evidently limit intake of nutritious food that contains toxins in accordance with the amount of a particular toxin they can detoxify (Provenza, 1996; Wang and Provenza, 1997). It seems clear that learning can play a crucial role in the acquisition of a generalist ruminant's feeding behavior, so that experienced ruminants are able to eat from a wide array of plants without ingesting a harmful dose of any particular toxin (Provenza and Balph, 1987).

The rumen environment of the goats responded rapidly to the administration of free oxalic acid. Increments in rates of degradation occurred within a short period after the beginning of the oral dosing, thus protecting the host animal from detrimental postingestive consequences of oxalic acid in the feed, in agreement with previous studies (Allison et al., 1977; Duncan et al., 1997). It was expected that the physiological impact of the oxalic acid consumed in the food would be reduced in adapted goats. Thus, adapted animals would be less influenced by aversions to oxalic acid-paired flavors than nonadapted goats. However, physiological adaptation did not influence dietary choice in this experiment since both adapted and nonadapted animals showed similar preferences during the conditioning period. This may have been due to the speed with which the rumen microflora adapted to oxalic acid, with evidence for adaptation even within the two-day feeding bouts. This was evidently sufficient to prevent marked differences in the physiological impact of ingested oxalic acid between adapted and nonadapted groups, with no evidence for an effect of adaptation regime on plasma calcium concentrations. The results emphasize the adaptability of the rumen environment and indicate that increased rates of intraruminal oxalic acid degradation in response to oxalic acid consumption may be sufficiently rapid to prevent variation in behavioral responses to oxalic acid ingestion between physiologically naive and adapted animals. The lack of difference in toxin tolerance threshold, as a determinant of intake limitation (Wang and Provenza, 1997), between adapted and nonadapted animals also supports this supposition. Further work is required to determine whether these findings have general applicability; it may be that for aversion agents requiring longer-term physiological adaptation, the degree of adaptation may influence the development of aversions.

The level of inclusion of oxalic acid in the hay administered to the animals (60 mmol/kg) was not sufficiently high to induce strong conditioned aversions. The level was chosen on the basis of concentrations found in plants that contain this compound, together with potential intake rates (Libert and Franceschi, 1987), in order to present the animals with a situation analogous to that encountered in natural foraging environments.

Animals showed a chronic mild hypocalcemia that was presumably the result of the oxalic acid ingestion, and this is supported by the fact that plasma calcium concentrations were marginally lower in samples collected immediately after the animals had received the diet associated with oxalic acid. The mild

nature of the hypocalcemia observed may have been due in part to the calcium content of the experimental diets. The medium- to low-quality alfalfa hay used in the experimental diets contained approximately 7.8 g Ca/kg, a proportion of which would have been available for reaction with oxalic acid to form calcium oxalate in the rumen. During the days that the experimental diets were offered, the animals would have consumed approximately 6.2 g of Ca and 6.7 g oxalic acid (concentrations of approximately 7.8 and 8.3 g/kg, respectively). Although some of the calcium in the hay (25–50%) would not have been available to the animals (Ward et al., 1979), it can be seen that the active component of the consumed oxalic acid (that not immediately bound by Ca in the rumen) would have been substantially less than the total amount consumed. Nevertheless, the amount of oxalic acid escaping precipitation by calcium in the digestive tract or degradation by rumen microbes was evidently sufficient to depress plasma calcium and elicit an aversion in the animals under study.

Previous work has shown that ruminants are able to develop mild conditioned food aversions to natural detrimental plant components when artificially administered concurrently with the experimental diet (Kyriazakis et al., 1997). The experimental reported here extended these findings to show that a similar phenomenon occurs in the more natural circumstances of incorporation of the aversive compound into the diet. The experiment further demonstrated the adaptability of ruminant herbivores when presented with potentially toxic food sources: conditioned food aversions were quick to develop (after one two-day feeding bout) and quick to disappear in the absence of reinforcement.

The mild conditioned aversion demonstrated in the current experiment was not modulated by prior physiological adaptation to the aversive compound. Modulation might, however, be important where more extreme CFAs are developed and where physiological adaptation is more long-term in nature and results in significant protection of the animal from toxic consequences.

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SYSTEMIC INDUCTION OF ALLELOCHEMICALS IN GLANDED AND GLANDLESS ISOGENIC COTTON BY *Spodoptera exigua* FEEDING

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Abstract—Induction of systemic resistance to feeding of beet armyworm, *Spodoptera exigua*, was investigated in two isogenic lines of Stoneville 213 cotton, *Gossypium hirsutum*, that differed in the presence of pigment glands. In laboratory bioassays, larvae strongly preferred to feed on glandless cotton plants when presented a choice between undamaged terminal leaves of undamaged glanded and glandless plants. Feeding damage inflicted by *S. exigua* larvae on the two oldest leaves of glanded plants seven days prior to feeding bioassays caused larvae to prefer by 33-fold the undamaged terminal foliage from undamaged plants compared to that from damaged plants. Feeding damage on glandless plants caused only a 2.6-fold greater preference for terminal foliage from undamaged plants over foliage from previously damaged plants. Extracts of terminal foliage from glanded cotton damaged seven days earlier had significantly greater quantities of terpenoid aldehydes (hemigossypolone, gossypol, and heliocides) than did foliage from undamaged glanded plants. Terpenoid aldehydes were undetectable in extracts of both undamaged and previously damaged glandless plants. The profile of volatile compounds collected from the headspace of mechanically damaged terminal leaves of undamaged glanded and glandless plants differed. Both cotton isolines released large quantities of lipoxygenase products (hexenyl alcohols, acetates, and butyrates), but glandless plants released only small amounts of mono- and sesquiterpenes compared to glanded plants. Glandless plants damaged seven days prior to volatile collection released significantly greater quantities of lipoxygenase products, β-ocimene, and α- and β-farnesene than did undamaged glandless plants. Previously damaged glanded plants released significantly greater quantities of all mono- and sesquiterpenes and hexenyl acetates and butyrates, but not alcohols. The relative importance of volatile compounds

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versus terpenoid aldehydes in induced feeding deterrence in cotton to *S. exigua* larvae is still unclear.

Key Words—Beet armyworm, *Spodoptera exigua*, cotton, *Gossypium hirsutum*, heliocides, gossypol, terpenes, volatiles, glands, feeding deterrent, induced defense, systemic response, plant-insect interaction.

INTRODUCTION

Cultivated Upland cotton, *Gossypium hirsutum* L., and all other species in the tribe Gossypieae of the Malvaceae are characterized by their ability to produce lysigenous pigment glands in vegetative and reproductive tissues (Fryxell, 1968). In green tissues of *G. hirsutum*, these glands contain gossypol and biosynthetically related terpenoid aldehydes that have been shown to have toxic, antinutritive, and feeding deterrent effects on many generalist-feeding lepidopterans, such as *Heliothis virescens* (F.), *Helicoverpa zea* (Boddie), *Trichoplusia ni* (Hübner), *Spodoptera exigua* Hübner, and *Spodoptera littoralis* Boisduval (Bottger and Patana, 1966; Shaver and Lukefahr, 1969; Lukefahr et al., 1977; Meisner et al., 1977; Stipanovic et al., 1977). The glands also contain volatile mono- and sesquiterpenes (Elzen et al., 1985). While high terpenoid aldehyde content is beneficial for plant resistance to insects, it is detrimental to the use of seed as a human or nonruminant animal food source and as an oil source (Cherry and Leffler, 1984). In the 1960s, plant breeders developed glandless varieties to overcome this problem. However, these varieties were attacked by generalist insects that were previously only minor pests of cotton (Bottger et al., 1964; Jenkins et al., 1966).

In previous studies, we demonstrated that glanded cotton plants responded systemically to feeding by larvae of *S. exigua* and *S. littoralis*. Terminal, undamaged leaves from plants previously damaged by larvae contained much greater quantities of terpenoid aldehydes (hemigossypolone, gossypol, and heliocides) than control plants (McAuslane et al., 1997), and were less preferred in feeding bioassays (Alborn et al., 1996; McAuslane et al., 1997). We suggested that feeding deterrence was associated with increased concentrations of terpenoid aldehydes in pigment glands of damaged plants. However, we did not measure other terpenes that occur in pigment glands. It is known that cotton plants that have been fed on by lepidopteran larvae respond systemically by emitting volatile terpenes in greater quantities and in different blends (Röse et al., 1996). Similarly induced volatile compounds from corn attract natural enemies of the larvae (Turlings et al., 1990). While it has been suggested that volatile terpenes may also influence larval feeding behavior (Loughrin et al., 1995), there has been little research to substantiate this suggestion.

The purpose of the studies described here was to investigate further the

role of the pigment gland contents in the cotton plant's systemic response to insect feeding. We hypothesize that the previously observed inducible feeding deterrence in glanded cotton was due to greater quantities of allelochemicals in pigment glands. To test this, we obtained two isogenic lines of cotton, one glanded with the genotype $Gl_2Gl_2Gl_3Gl_3$ and one glandless with the genotype $gl_2gl_2gl_3gl_3$ (McCarty et al., 1996); two genes, gl_2 and gl_3, are responsible for the glandlessness trait in cotton leaves (McMichael, 1960). If feeding deterrence is associated solely with pigment glands, the glandless isoline should be unable to mount a systemic response to feeding by *S. exigua* larvae. We compared larval response in feeding choice bioassays to foliage from damaged or undamaged glanded and glandless plants. We also quantified terpenoid aldehydes in extracts of foliage and quantified volatile chemicals collected from the headspace of glandless and glanded plants.

METHODS AND MATERIALS

Plants. Seeds of Stoneville 213 (glanded) and a Stoneville 213 glandless isogenic line were planted in a greenhouse in 4-liter pots filled with a commercial potting mix (MetroMix 220, Grace Sierra, Milpitas, California). The greenhouse was illuminated with natural light, and the light cycle ranged from 14L:10D to 12L:12D (80 ± 10% relative humidity, and 30 ± 10°C). Plants were watered as necessary and fertilized once at the first-true-leaf stage with 5 ml of slow-release-formulation fertilizer (Osmocote 14-14-14, Scotts-Sierra, Marysville, Ohio).

Insects. Beet armyworm larvae, *S. exigua*, were reared on an artificial diet, based on pinto beans, according to established methods (King and Leppla, 1984). Third instars or early fourth instars were used in all experiments and were starved for 4 hr prior to experiments to encourage immediate feeding.

Feeding Preference of S. exigua *Between Undamaged Glanded and Glandless Stoneville 213.* Leaves from undamaged glanded and glandless cotton plants were paired in a feeding bioassay to determine the effects of the glanded trait on feeding preference on *S. exigua* larvae. Plants used in bioassays were at the five- to seven-true-leaf developmental stage. The two youngest leaves (Y1 and Y2) of glanded and glandless plants were cut from the plants and their petioles immediately wrapped in wet cotton and inserted into water-filled 1.5-ml Eppendorf vials. The four leaves were photocopied and then arranged symmetrically in a circular clear plastic container (19-cm diameter × 10-cm height) with a lid. Four third-instar larvae were placed in the center of the leaf array. After 24 hr, larvae were removed and leaves were again photocopied. Leaf photocopies were scanned and imported into an imaging program (ImagePC beta version 1, Scion Corporation, Frederick, Maryland) where leaf area eaten and leaf area

remaining were quantified. Ten replicate bioassays were conducted simultaneously.

Feeding Preferences in Glanded and Glandless Stoneville 213 After Damage by S. exigua *Feeding.* A second set of experiments was conducted to determine whether previous larval feeding would induce feeding deterrence in both glanded and glandless Stoneville 213 cotton plants. Plants used in these experimental had six to seven true leaves.

Two fourth-instars larvae were placed on each of the two oldest leaves of 18 glanded plants and 18 glandless plants. Leaves with larvae were enclosed in perforated plastic bags (Ziploc vegetable bags, DowBrands, Indianapolis, Indiana) sealed with a pipe cleaner twisted gently around the leaf petiole (i.e., damaged treatment). The two oldest leaves of an additional 18 glanded and 18 glandless plants received plastic bags and a pipe cleaner but no larvae (i.e., undamaged control). Larvae and bags were removed 48 hr later. One week after larvae were removed, 10 replicate feeding bioassays were set up. The additional eight replicates of glanded and glandless plants were prepared for terpenoid aldehyde analysis by high-performance liquid chromatography (HPLC).

Feeding bioassays were conducted as previously described. The two youngest leaves (Y1 and Y2) were cut from damaged and control glanded plants. The four leaves were arranged symmetrically in a clear plastic rectangular box (24 cm long × 32 cm wide × 11 cm high) with a lid and three fourth instars were placed in the center. Larvae were removed after 24 hr and leaves were measured. Bioassays comparing larval feeding on foliage from damaged and control glandless plants were conducted simultaneously.

Quantification of Terpenoid Aldehydes in Glanded and Glandless Stoneville 213. Pooled terminal leaves (Y1 and Y2) from control and damaged glanded and glandless plants ($N = 8$ plants per treatment–isoline combination) were prepared separately for HPLC analysis. Leaves frozen at −70°C overnight were lyophilized and ground through a 40-mesh brass screen to a uniform powder. For each glanded sample, 100 mg of dried foliage was extracted with 3 ml hexane–ethyl acetate (3 : 1) and 0.2 ml 10% HCl in water. Samples were extracted a second time with 2 ml hexane–ethyl acetate (3 : 1). For each glandless sample, 500 mg of dried foliage were extracted. The combined organic fraction was concentrated to near dryness with N_2 and 1 ml of HPLC solvent was added [19.5% ethanol, 5.4% methanol, 14.2% isopropyl alcohol, 23.6% acetonitrile, 27.0% water, 4.2% ethyl acetate, 6.0% dimethylformamide, and 0.1% phosphoric acid (modified from Stipanovic et al., 1988)]. After 10 min in an ultrasonic bath, the solid residue was filtered through a 0.45-μm nylon filter. Extracts of glandless plants only were concentrated again to near dryness and redissolved in 50 μl HPLC solvent (i.e., 10× concentration). Samples were analyzed on a Shimadzu LC-600 liquid chromatograph equipped with UV–visible spectrophotometric detector set at 272 nm, and a 25-cm × 4-mm-ID C-18 column (ODS2

8/5 packing, SGE, Austin, Texas). HPLC was run in the isocratic mode at ambient temperature with a flow rate of 1.25 ml/min. Terpenoid aldehydes were identified by reference to retention times of authentic compounds (McAuslane et al., 1997) and quantified in terms of gossypol equivalents with commercial gossypol (Sigma Chemical Company, St. Louis, Missouri). Five replicate extractions were made for each treatment–isoline combination and two replicate injections of 20 μl/extraction were made onto the column.

Volatiles Released by Glanded and Glandless Stoneville 213 Cotton After Damage by S. exigua *Feeding.* Plants were damaged by confining two fourth instars on each of the two oldest leaves of seven- to eight-leaf-stage plants, as described above. Control plants received plastic bags on the two lower leaves, but no larvae. Larvae were allowed to feed for 48 hr, after which time larvae and bags were removed from all plants. Volatiles were collected from plants one week after larvae were removed to determine whether previous larval feeding induces systemic release of volatiles from both glanded and glandless Stoneville 213.

Volatiles were collected simultaneously from equal numbers of damaged and control glanded and glandless plants. The undamaged terminal portion of each plant, bearing leaves corresponding to Y1 and Y2 used in feeding bioassays, was excised using a razor blade. The petiole was immediately wrapped in wet cotton and inserted into a 1.5-ml Eppendorf plastic vial filled with water. Steel forceps were used to make two crimps on the Y2 and one on the Y1 (area of damage per crimp = 0.66 $\pm$ 0.06 cm^2, mean $\pm$ SD). The purpose of this mechanical damage was to mimic the release of volatiles that a feeding larva might cause. Plant terminals were immediately placed in glass volatile collection chambers measuring 3 cm diam. $\times$ 20 cm long (described in detail in Turlings et al., 1991). Using a push–pull system, purified and humidified air passed over the plant material at 300 ml/min. Volatiles were collected over a 3-hr period (13:30 to 16:30 hr EST) onto cartridges filled with 25 mg Super-Q adsorbant (Alltech Associates, Deerfield, Illinois). The volatile collection system was set up in climate-controlled room under a bank of two 400-W sodium lamps and one 400-W metal halide lamp providing ca. 280 μmol photons/m^2/sec at chamber height.

In the laboratory, volatiles were eluted from the adsorption cartridges with 150 μl methylene chloride, to which was added 4000 ng nonyl acetate in 50 μl solvent as an internal standard. One-microliter samples were injected into a HP 5890 gas chromatograph (GC) equipped with a HP 7673 autoinjector operated in the splitless mode. Samples were analyzed on a fused silica DB1 capillary column (50 m $\times$ 0.25 mm ID with 0.25-μm-thick film) (Quadrex Corporation, New Haven, Connecticut). The oven was maintained at 60°C for 2 min and then increased at a rate of 4°C/min to a final temperature of 180°C and maintained at 180°C for 10 min. The flame ionization detector was set at 275°C.

The carrier gas, helium, was adjusted to a flow rate of 19 cm/sec. Volatile compounds were identified via comparison of GC retention times and mass spectra with those of authentic compounds or spectra in a spectral library data base.

Statistical Analyses. Leaf area eaten in feeding bioassays was expressed directly as area eaten in square centimeters and as the proportion of each leaf eaten. Analysis of variance was performed by the MIXED procedure (SAS Institute, 1995) on area data that were transformed via log $(x + 0.1)$ to stabilize variability across predicted means. Proportion eaten data were transformed via arcsine $-\sqrt{x}$. Means were separated when appropriate with Tukey-Kramer tests (SAS Institute, 1995) with a significance level of $\alpha = 0.05$. Untransformed means and standard errors of the mean are presented in the tables. Quantities of terpenoid aldehydes were expressed in gossypol equivalents and were compared between control and damaged plant extracts by t tests (TTEST procedure, SAS Institute, 1995). Quantities of volatile chemical released from damaged and control plants were compared by Mann-Whitney U tests (NPAR1WAY procedure, SAS Institute, 1995).

RESULTS

Feeding Preference of S. exigua *Between Undamaged Glanded and Glandless Stoneville 213.* Third instars of *S. exigua* consumed significantly more foliage from undamaged glandless plants than from glanded plants ($F = 88.99$; $df = 1,27$; $P < 0.001$) (Table 1) when given a choice between the two isolines. Leaf age (Y1 or Y2) was also a significant source of variation ($F = 25.56$; $df = 1,27$; $P < 0.001$); however, there was a significant leaf age × isoline

TABLE 1. FEEDING PREFERENCE OF *S. exigua* LARVAE WHEN PRESENTED A CHOICE AMONG LEAVES FROM UNDAMAGED GLANDED (GL) AND GLANDLESS (gl) STONEVILLE 213 COTTON PLANTS

		Mean ± SEM[b]	
Isoline	Leaf[a]	Leaf area consumed (cm^2)	Proportion of leaf consumed
GL	Y1	0.45 ± 0.18a	0.010 ± 0.004a
	Y2	2.71 ± 0.63b	0.044 ± 0.015a
gl	Y1	6.86 ± 1.60c	0.21 ± 0.04b
	Y2	9.54 ± 1.68c	0.13 ± 0.02b

[a]Youngest leaf (Y1) or second youngest leaf (Y2) on plant.
[b]Means separated by Tukey-Kramer test at $P = 0.05$ (SAS Institute, 1995).

interaction ($F = 10.49$; $df = 1,27$; $P = 0.0032$). Larvae ate significantly less of the youngest leaf (Y1) of glanded plants than of the second youngest leaf (Y2); in contrast, larvae ate similar amounts of the Y1 and Y2 leaves of the glandless plants (Table 1). The proportion of each leaf consumed was significantly affected by a leaf age × isoline interaction ($F = 12.78$; $df = 1,27$; $P = 0.0013$) (Table 1). Cotton isoline was significant ($F = 78.74$; $df = 1,27$; $P < 0.001$) but leaf age was not. We can explain the interaction by the fact that on glanded cotton larvae consumed a smaller proportion of the Y1 leaf than the Y2 leaf (Table 1). In contrast, larvae consumed a greater proportion of the Y1 leaf than the Y2 leaf on glandless cotton.

Feeding Preferences in Glanded and Glandless Stoneville 213 After Damage by S. exigua *Feeding.* Larvae consumed significantly less foliage from glanded plants damaged seven days previously than from control glanded plants (Table 2) ($F = 75.89$; $df = 1,27$; $P < 0.001$). In addition, larvae ate significantly less of the Y1 leaves than the Y2 leaves ($F = 67.18$; $df = 1,27$; $P < 0.001$). There was a significant treatment × leaf age interaction ($F = 16.97$; $df = 1,27$; $P < 0.001$). Both main effects and the treatment × leaf age interaction explained significant amounts of the variability in the proportion of each leaf eaten (treatment: $F = 58.34$; $P < 0.001$; leaf age: $F = 30.29$; $P < 0.001$; treatment × leaf age: $F = 14.23$; $P < 0.001$; $df = 1,27$).

Larvae consumed significantly less foliage from previously damaged glandless cotton than from control glandless cotton (Table 2) ($F = 28.98$; $df = 1,27$;

TABLE 2. FEEDING PREFERENCES OF *S. exigua* LARVAE WHEN PRESENTED CHOICES AMONG UNDAMAGED LEAVES FROM CONTROL AND PREVIOUSLY DAMAGED GLANDED (GL) OR GLANDLESS (gl) STONEVILLE 213 COTTON PLANTS

			Mean ± SEM[b]	
Isoline	Treatment	Leaf[a]	Leaf area consumed (cm^2)	Proportion of leaf consumed
GL	Control	Y1	0.60 ± 0.32b	0.020 ± 0.008a
	Control	Y2	13.9 ± 2.9c	0.14 ± 0.03b
	Damaged	Y1	0.003 ± 0.002a	0.0001 ± 0.0001a
	Damaged	Y2	0.43 ± 0.21b	0.004 ± 0.002a
gl	Control	Y1	4.67 ± 1.10b	0.17 ± 0.03b
	Control	Y2	35.5 ± 10.6c	0.35 ± 0.06c
	Damaged	Y1	0.66 ± 0.55a	0.02 ± 0.02a
	Damaged	Y2	14.7 ± 2.1c	0.18 ± 0.03b

[a] Youngest leaf (Y1) or second youngest leaf (Y2) on plant.
[b] Means within a cotton isoline separated by Tukey-Kramer test at $P = 0.05$ (SAS Institute, 1995).

$P < 0.001$). Leaf age and treatment × leaf age interaction were also significant sources of variability (leaf age: $F = 108.61$; $P < 0.001$; interaction: $F = 8.05$; $P = 0.0085$; $df = 1,27$). Larvae ate a significantly smaller proportion of leaves from damaged plants and from the youngest leaf (treatment: $F = 24.94$; $P < 0.001$; leaf age: $F = 30.97$; $P < 0.001$; $df = 1,27$). However, there was no significant treatment × leaf interaction for the proportion of each leaf eaten.

The larvae in the glandless bioassays consumed significantly more foliage in 24 hr than did the larvae in the glanded bioassays (average of 55.5 ± 10.4 cm^2 in glandless bioassays versus 15.0 ± 3.0 cm^2 in glanded bioassays) (t test; $P < 0.001$; $df = 18$). This difference was not influenced by total leaf area offered in each bioassay (average of 232 ± 20 cm^2 in glandless bioassays and 248 ± 20 cm^2 in glanded bioassays).

Quantification of Terpenoid Aldehydes in Glanded and Glandless Stoneville 213. The youngest undamaged leaves of glanded plants that had been damaged seven days earlier by larval feeding contained significantly more hemigossypolone, gossypol, and heliocides than control plants (Table 3). H_1 and H_4

TABLE 3. TERPENOID ALDEHYDE CONTENT OF UNDAMAGED YOUNG LEAVES OF GLANDED STONEVILLE 213 PLANTS WHOSE TWO OLDEST LEAVES WERE UNDAMAGED (CONTROL) OR DAMAGED 7 DAYS PREVIOUSLY BY *S. exigua*

Terpenoid aldehyde[a]	Mean ± SEM		Increase (%)	P^b
	Control	Damaged		
	Gossypol equivalents (ng/mg dried foliage)			
HGQ	3062 ± 26	7613 ± 108	149	<0.001
G	191 ± 18	428 ± 16	124	<0.001
H_1	321 ± 5	1449 ± 40	351	<0.001
H_4	94 ± 11	552 ± 21	487	<0.001
H_2	780 ± 13	1109 ± 26	42	<0.001
H_3	317 ± 5	460 ± 10	45	<0.001
TTA	5306 ± 54	11611 ± 184	119	<0.001
	Ratios among terpenoid aldehydes			
$H_1 + H_4/H_2 + H_3$	0.38 ± 0.01	1.28 ± 0.01		<0.001
$HGQ/(H_1 + H_2 + H_3 + H_4)$	2.39 ± 0.05	2.14 ± 0.04		0.0011

[a] Abbreviations of terpenoid aldehydes: HGQ, hemigossypolone, G, gossypol, H_1–H_4, heliocides 1–4, TTA, total terpenoid aldehydes.

[b] t tests for a significant difference between extracts of control and damaged plants were performed after ensuring homogeneity of variance (PROC TTEST, SAS Institute, 1995).

increased the most of all terpenoid aldehydes measured, by 351% and 487%, respectively. The ratio of ocimene-derived heliocides (H_1 + H_4) to myrcene-derived heliocides (H_2 + H_3) differed significantly in damaged and control plants (Table 3). The ratio in control plants was heavily biased toward H_2 and H_3 (0.38), whereas the ratio in damaged plants was heavily biased toward H_1 and H_4 (1.28). In addition, the ratio of hemigossypolone to all heliocides was significantly lower in foliage from damaged plants than from control plants (Table 3).

The HPLC chromatograms of damaged glandless plants did not differ noticeably from those of control plants (data not shown). Terpenoid aldehydes were not detected above baseline noise in the extracts despite a 50-fold greater concentration of the extracts and attenuating the detector response by eightfold less compared to glanded extracts. Many small peaks were observed, but peak shapes and retention times did not match those of the six terpenoid aldehydes of interest.

Volatiles Released by Glanded and Glandless Stoneville 213 Cotton after Damage by S. exigua *Feeding.* Twenty-nine volatile compounds were identified in the headspace of mechanically damaged glanded and glandless Stoneville 213 (Table 4). Both glanded and glandless plants that had been damaged seven days earlier by larval feeding released significantly greater amounts of (*Z*)-3-hexenyl acetate, hexyl acetate, (*Z*)-3-hexenyl butyrate, and (*Z*)-3-hexenyl-2-methyl butyrate (lipoxygenase products) upon mechanical damage than did control plants with the same mechanical damage. In addition, damaged glandless plants released greater quantities of other lipoxygenase products [i.e., (*Z*)-3-hexenal, (*E*)-3-hexenol and (*Z*)-3-hexenol] than did control plants. Control glandless plants released very small quantities of terpenoid compounds compared to glanded plants, with the exception of ocimene, (*E*)-β-farnesene, and (*E*,*E*)-α-farnesene (Table 4). A greater release of mono- and sesquiterpenes was not induced in damaged glandless plants except for the three terpenes already mentioned. In contrast, control glanded plants released large amounts of terpenes, and plants that had sustained feeding damage seven days earlier released significantly greater amounts of all terpenes quantified (Table 4). Other compounds identified and quantified did not differ significantly between damaged and control plants for either glanded or glandless plants (Table 4).

DISCUSSION

In our laboratory feeding study, larvae of *S. exigua* preferred to feed on terminal foliage from undamaged glandless Stoneville 213 cotton plants than on foliage from undamaged glanded plants (Table 1). Glandless cotton is more susceptible than glanded cotton to herbivory by various insects in the field,

TABLE 4. VOLATILES RELEASED FROM MECHANICALLY DAMAGED TERMINALS EXCISED FROM GLANDED AND GLANDLESS STONEVILLE 213 COTTON PLANTS UNDAMAGED (CONTROL) OR FED ON BY *S. exigua* LARVAE 7 DAYS PREVIOUSLY (DAMAGED)

	Median (range) ng released/3 hr[a]					
	Glandless			Glanded		
Compound	Control	Damaged	P^b	Control	Damaged	P
Lipoxygenase products						
(*Z*)-3-Hexenal	174 (130–385)	740 (503–1570)	*	272 (104–363)	362 (201–895)	
(*E*)-2-Hexenal	128 (74–224)	254 (139–413)		143 (71–227)	173 (121–361)	
(*E*)-3-Hexenol	6 (5–10)	19 (12–28)	*	7 (6–9)	12 (4–26)	
(*Z*)-3-Hexenol	844 (527–1329)	3298 (1943–6031)	*	668 (605–1121)	829 (665–1184)	
(*E*)-2-Hexenol	45 (33–69)	121 (41–316)		37 (20–65)	54 (34–149)	
(*Z*)-2-Hexenol	46 (30–77)	86 (47–112)		37 (27–62)	59 (32–118)	
(*Z*)-3-Hexenyl acetate	1711 (1273–3405)	11228 (5597–15418)	*	2159 (762–2886)	4778 (3165–10404)	*
Hexyl acetate	19 (16–32)	95 (41–224)	*	18 (7–45)	49 (37–222)	*
(*Z*)-3-Hexenyl butyrate	157 (93–218)	996 (734–1201)	*	80 (45–207)	214 (104–373)	*
(*E*)-2-Hexenyl butyrate	11 (3–23)	41 (21–55)	0.06	14 (0–16)	21 (13–38)	0.06
(*Z*)-3-Hexenyl 2-methyl butyrate	31 (20–49)	94 (85–174)	*	17 (11–29)	38 (29–70)	*
(*E*)-2-Hexenyl 2-methyl butyrate	66 (51–79)	106 (35–212)		60 (37–77)	74 (57–163)	
Jasmone	75 (30–144)	173 (88–302)		29 (7–75)	49 (20–140)	

Monoterpenes						
α-Pinene	270 (214–417)	235 (190–355)		2629 (1643–3601)	8962 (2940–12706)	*
β-Pinene	nd	nd		400 (210–562)	1457 (460–2120)	*
Myrcene	50 (30–178)	82 (59–122)		1333 (891–2250)	5517 (1909–9071)	*
Limonene	16 (7–33)	13 (8–30)		196 (119–284)	637 (247–1025)	*
β-Ocimene	683 (465–1360)	1620 (721–2011)	0.06	596 (349–1546)	3692 (1895–4790)	*
Sesquiterpenes						
β-Caryophyllene	5 (0–158)	10 (8–22)		957 (577–1649)	5213 (1932–7209)	*
(*E*)-β-Farnesene	7 (0–10)	105 (53–155)	*	17 (9–37)	114 (79–140)	*
α-Humulene	2 (0–40)	7 (4–7)		279 (170–430)	1404 (508–2087)	*
(*E*,*E*)-α-Farnesene	37 (25–78)	120 (87–363)	*	23 (17–61)	70 (36–162)	*
γ-Bisabolene	2 (0–55)	6 (0–8)		310 (127–656)	1919 (869–2292)	*
β-Bisabolol	2 (0–28)	nd		182 (114–332)	549 (422–858)	*
Others						
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	327 (239–386)	429 (266–768)		160 (35–216)	349 (141–651)	0.06
Indole	43 (26–75)	90 (28–153)		25 (6–39)	28 (9–63)	
(*E*,*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	81 (72–510)	36 (22–177)		42 (9–188)	61 (25–683)	

[a] Median of collections from four glandless plants and six glanded plants.
[b] Asterisks indicate significantly different amounts of volatile compounds between control and damaged plants within an isoline (Mann-Whitney U test, $P < 0.05$).

including *S. exigua*, *H. zea*, *Alabama argillacea* (Hübner), and leaf-chewing beetles (Bottger et al., 1964; Jenkins et al., 1996). In no-choice field studies, *H. zea* larvae caged on glandless Stoneville 213 plants damaged significantly more leaves than larvae caged on glanded Stoneville 213 plants (Zummo et al., 1983). In choice tests conducted over a five-day period in a greenhouse, second instars of *S. exigua* defoliated all glandless seedlings before moving onto adjacent glanded plants (Bottger et al., 1964). Thus, it is well established that the presence or absence of pigment glands in cotton influences the feeding behavior of generalist-feeding lepidopteran insects.

Feeding damage by *S. exigua* larvae seven days prior to bioassays induced feeding deterrence in undamaged terminals of Stoneville 213 glanded cotton (Table 2), similar to our findings with Deltapine 90 (Alborn et al., 1996; McAuslane et al., 1997). However, contrary to our expectations, some feeding deterrence was also induced in glandless Stoneville 213 plants; foliage from damaged glandless plants was 2.6-fold less palatable than foliage from control plants. While this reduction was very much less than the 33-fold reduction in palatability in damaged glanded plants, there is still evidence that feeding deterrence can be induced in glandless cotton.

The induction of feeding deterrence in glandless plants cannot be explained by terpenoid aldehydes that were not detected in either control or damaged glandless Stoneville 213. Some studies have reported amounts of "gossypol" (estimated by the phloroglucinol reaction) in leaves of field-grown glandless Stoneville 213 ranging from 37% to 74% of that of glanded Stoneville 213 (Hedin et al., 1991; McCarty et al., 1996). However, the phloroglucinol reaction is a nonspecific reaction for terpenoid aldehydes (Bell et al., 1974), and these data probably represent quantification of other terpenoid aldehydes, in addition to gossypol. Quantification of individual terpenoid aldehydes in Stoneville 213 by HPLC revealed that buds of field-grown glandless plants had small quantities of terpenoid aldehydes, ranging from 14% (gossypol and H_2) to 25% (hemigossypolone) of that of glanded plants (Hedin et al., 1991). Other researchers have indicated that terpenoid aldehydes are never detected in leaves of healthy glandless cotton plants (Bell and Stipanovic, 1976). However, glandless plants can produce terpenoid aldehydes locally in stem or hypocotyl tissue infected by pathogenic fungi [gossypol (Bell, 1967); gossypol, hemigossypol, desoxyhemigossypol, and related methoxylated terpenoid aldehydes (Hunter et al., 1978)]. It is possible that the field-grown cotton sampled in the previous experiments was pathogen-infected, and therefore produced quantifiable amounts of terpenoid aldehydes. The plants used in our study were greenhouse grown and insect- and pathogen-free. Why fungal infection induces localized production of terpenoid aldehydes in stems and hypocotyls of glandless plants while herbivore damage did not induce systemic production of terpenoid aldehydes in leaves in our study is an intriguing, but unanswered, question.

In contrast to glandless plants, HPLC analysis revealed strong induction

of heliocides, gossypol, and hemigossypolone in undamaged leaves of damaged glanded Stoneville 213 plants (Table 3). These results were similar to Deltapine 90 except that we reliably detected heliocide H_4 in Stoneville 213 and found that its concentration increased the most of all heliocides measured; H_4 was often undetectable in undamaged Deltapine 90 (McAuslane et al., 1997). Heliocide H_1 was next most strongly induced heliocide in Stoneville 213 and was the most induced in Deltapine 90. Similar to our results with Deltapine 90, damaged Stoneville 213 plants had a higher ratio of $H_1 + H_4$ to $H_2 + H_3$, and the ratio of hemigossypolone to heliocides was characteristic of the ratio normally found in older leaves, with less hemigossypolone and relatively more heliocides (Bell et al., 1978).

Terpenoid aldehydes were not the only terpenes induced in previously damaged plants in our experiments. Volatile terpenes were released in greater quantity from mechanically damaged glandless and glanded plants that had been damaged by larvae seven days previously compared to mechanically damaged control plants (Table 4). These results indicate that larvae in the feeding bioassays with glandless cotton faced greater quantities of ocimene and α- and β-farnesene from damaged plants than from control plants. Larvae in feeding bioassays with glanded cotton confronted greater quantities of all mono- and sesquiterpenes in damaged plants compared to controls. Elzen et al. (1985) found that solvent extracts of Stoneville 213 leaves contained 118-fold greater quantities of terpenes than extracts of Stoneville 209 (a glandless isoline of Stoneville 213). In our experiments control glanded plants released 6.4-fold more terpenes than control glandless plants during a 3-hr period (6922 ng vs. 1074 ng), and damaged glanded plants released 13.4-fold more terpenes (29,534 ng vs. 2198 ng) than damaged glandless plants (Table 4). These data strongly suggest that, while glandless plants cannot store volatile terpenes because they have no glands, they are still capable of synthesizing and releasing relatively large amounts of certain terpenes (ocimene and α- and β-farnesene) after herbivore damage.

It is of interest that these same acyclic terpenes (ocimene and α- and β-farnesene) are emitted in a diurnally cyclic fashion (Loughrin et al., 1994) and are synthesized de novo in Deltapine 90 cotton after herbivore damage (Paré and Tuminson, 1997). Linalool and the homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, also shown to be synthesized de novo (Paré and Tumlinson, 1997) and emitted cyclically (Loughrin et al., 1994), were not found in significantly higher amounts in glanded or glandless plants damaged seven days previously. The fact that our plants were sampled one week after damage while those in the studies cited above were sampled during larval feeding or soon after the end of feeding may account for the difference. Alternatively, Deltapine 90 and Stoneville 213 may differ in their response to herbivore damage.

Storage of mono- and sesquiterpenes by plants is often assumed to be a

defense against insect herbivory. However, other than for coniferous plants (Heikkenen and Hrutfiord, 1965; Sturgeon, 1979), data supporting this assumption are rare (Harborne, 1991). Leaf cutter ants, *Atta cephalotes*, were repelled by plants species whose leaves contained high amounts of β-ocimene, caryophyllene oxide, spathulenol, and caryophyllene (Hubbell et al., 1983). We detected only small amounts of the most potent of these terpenes, caryophyllene oxide (Howard et al., 1989), in both damaged and control glanded cotton, and we did not detect any spathulenol, even though it has been identified from Stoneville 213 cotton (Elzen et al., 1984). Sesquiterpene lactones are well known feeding deterrents in the Compositae (Harborne, 1991) but have not been identified from cotton.

A few studies have demonstrated effects of mono- and sesquiterpenes on lepidopteran feeding. Larvae of *H. virescens* grew more slowly on artificial diet fortified with caryophyllene or caryphyllene oxide than on control diet (Gunasena et al., 1988). It is not known, however, whether feeding behavior was different on diets incorporating these sesquiterpenes. Herbivory by lepidopteran larvae was reduced on individual trees in Brazil with higher amounts of caryophyllene relative to other terpenoids in their leaf resin (Langenheim et al., 1986), and caryophyllene incorporated into artificial diet deterred feeding by *S. exigua* (Langenheim et al., 1980). Larvae of *Spodoptera littoralis* consumed less artificial diet during a 2-hr or 5-hr period when it was infused with 0.5–1% (−)-carvone, a monoterpene in dill essential oil, compared to control artificial diet (Meisner et al., 1982). Finally α-pinene, β-pinene, 3-carene, and camphene were highly phagodeterrent to larvae of the gypsy moth, *Lymantria dispar* (Meisner and Skatulla, 1975). In all these studies, however, it is difficult to correlate amounts of chemicals in artificial diet that cause feeding deterrence with amounts of volatiles released over a 3-hr period.

In addition to volatile terpenes, both glandless and glanded herbivore-damaged plants released significantly greater amounts of lipoxygenase-derived volatiles after mechanical damage than did control plants (Table 4). Glanded cotton cultivars, Stoneville 213 (McAuslane, 1990) and Deltapine 90 (Loughrin et al., 1994, 1995; McCall et al., 1994; Röse et al., 1996), have been shown to produce large amounts of lipoxygenase products during active larval feeding or after mechanical damage. However, lipoxygenase products have not been quantified in glandless plants previously. The increase in lipoxygenase products in glanded and glandless damaged plants compared to control plants suggests that herbivore damage seven days earlier caused an increase in lipoxygenase activity in developing foliage, either through increased production or reduced catabolism. Higher lipoxygenase activity has been found in squares, but not terminal foliage, of glanded cotton plants damaged 72 hr previously by *H. zea* (Bi et al., 1997). It is not clear, however, whether the tissue sampled in that study was undamaged or had sustained feeding damage, as the larvae were not confined

to the lower portion of the plant. The same study suggested that other changes in oxidative enzymes that occurred in herbivore-damaged plants produced antinutritive effects on feeding larvae (Bi et al., 1997). However, one would expect similar induction of resistance in glanded and glandless cotton plants if resistance were associated with increased activities of oxidative enzymes rather than with pigment gland-associated terpenoid allelochemicals. Our data provide evidence that induction of the lipoxygenase system is similar in glandless and glanded Stoneville 213 plants. Lipoxygenase products may contribute to the 2.6-fold feeding deterrence in damaged glandless plants. Experiments are underway to determine whether induction of antibiotic resistance in glanded and glandless Stoneville 213 follows the pattern shown by antixenotic resistance (i.e., feeding deterrence).

In conclusion, we still cannot determine definitely why cotton plants subjected to larval feeding become deterrent to further larval feeding. There is more than a 10-fold greater deterrence in a glanded isoline after herbivore damage (with increased quantities of terpenoid aldehydes and volatiles emitted compared to undamaged controls) than in a glandless isoline (no increase in terpenoid aldehydes and only a slight increase in volatile emission). It is possible that feeding deterrence is due to a synergistic effect of volatile and nonvolatile terpenoids, as has been suggested for the toxic effects of cotton allelochemicals on *H. virescens* (Gunasena et al., 1988). Possibly other factors, associated mainly with glanded cultivars, that were not measured in this study are involved. The interesting phenomenon of systemically induced feeding deterrence in cotton needs further study.

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EFFECT OF SOIL SULPHUR LEVELS ON FEEDING PREFERENCE OF *Brevicoryne brassicae* ON BRUSSELS SPROUTS

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Abstract—Two cultivars of Brussels sprouts, *Brassica oleracea* var. *gemmifera* were grown in pots treated with nutrient solutions containing five different levels of soil sulfur. Three replicates, each with 10 pots, were infested with the alate form of the aphid, *Brevicoryne brassicae*. Another three replicates were not infested with aphids. Total apterae aphids were counted on each leaf of the infested plants four weeks later. At the same time all plants were assayed for total glucosinolates and free thiocyanates. The greatest number of aphids per leaf on infested plants was found for the highest levels of sulfur applied. The highest concentrations of total glucosinolates were found in the leaves of uninfested plants that received the highest levels of sulfur. The lowest level of total glucosinolates was found in the leaves of infested plants that received the highest amount of sulfur, and these leaves had the highest level of free thiocyanates. The results suggest that aphids were attracted in greatest numbers to leaves that had the highest synthesis of glucosinolates and that their presence resulted in reduced glucosinolate levels by a combination of a depression in metabolic rate and a stimulation in myrosinase activity.

Key Words—Sulfur, glucosinolate, thiocyanate, aphid, Brussels sprouts.

INTRODUCTION

The cabbage aphid, *Brevicoryne brassicae* L., is found worldwide and only feeds on plants in the Cruciferae family, such as cabbage, cauliflower, and

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Brussels sprouts. The insects aggregate in large clusters, usually on the undersides of the youngest leaves. Because of their rapid development time, asexual reproduction, and long reproductive life-span, aphids can complete up to 15 generations during the growing season. Their feeding damage can kill seedlings and, on mature plants, can cause the leaves to curl and turn yellow, stunt plant growth, and deform the developing heads.

The levels of secondary sulfur-containing metabolites, such as glucosinolates, in the tissues of cruciferous crops are reported to have a role in the chemical interaction between the plants and certain insect pests (Van Emden, 1972; Wolfson, 1982; Harborne, 1988; Niemeyer, 1990; Barlet and Williams, 1991). They can act as a feeding stimulant for some insects that specialize in feeding on the Cruciferae, such as *B. brassicae* (Van Emden, 1972), and as a feeding deterrent for other insects (Harborne, 1988).

A reduction in the level of soil sulfur is usually accompanied by a reduction in the levels of the glucosinolates and their enzymic breakdown products (Kaur et al., 1990; Booth et al., 1991; Walker and Booth, 1994; Williams et al., 1996). For some Cruciferae this has been associated with changes in their interaction with insects (Nault and Styer, 1972; Wolfson, 1982; Niemeyer, 1990). However, there do not appear to have been any studies reported on the insect feeding preference of *B. brassicae* on Brussels sprouts plants treated with different levels of sulfur.

This paper reports the effect of growing two cultivars of Brussels sprouts plants with different amounts of soil sulfur on the levels of total glucosinolates and free thiocyanates in the leaves and the feeding preference of the aphid, *B. brassicae*, when given a choice of plants grown with a range of soil sulfur.

METHODS AND MATERIALS

Seeds of Brussels sprouts, cultivars Roger and BS 085, were donated by Northrup King Pty. Ltd. They were treated with hot water (50°C) for 25 min to protect against seed-borne diseases (Meakins, 1979), and germinated at 22°C in vermiculite. Two weeks after germination, the seedlings were transferred to a commercial potting mix and grown for a further four to six weeks until the first true leaves appeared. Two seedlings per pot were then transplanted into 25-cm-diameter plastic pots containing a sterile sand/perlite mix (2:1, 9.6 kg dry weight) and kept in a glasshouse maintained at 23°C. Two weeks later, one plant was removed from each pot leaving the healthier plant remaining.

A standard nutrient solution containing no sulfur, based on Hewitt and Smith (1975), was supplemented with different amounts of 0.5 M K_2SO_4 (Figure 1) and adjusted to pH 7.0 with 0.5 M NaOH. The levels of sulfur used were determined from previous tests to give a significant increase in total glucosinolates. The highest level used (15 ppm) equates to about 88 kg/ha applied in

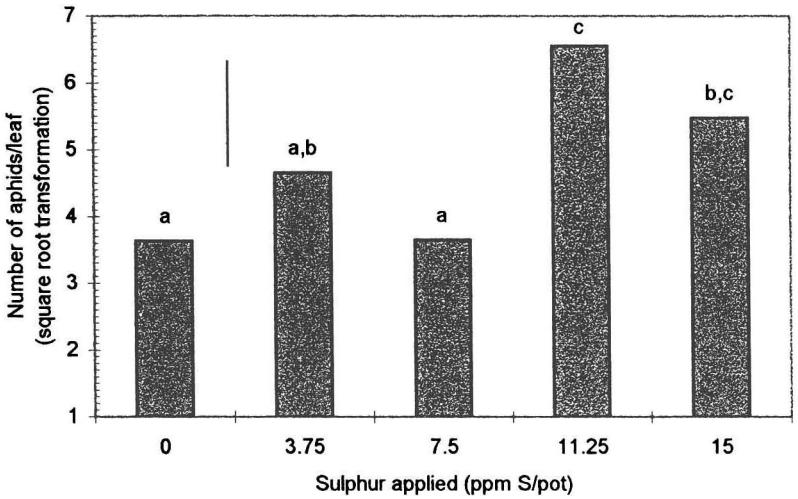

FIG. 1. Square root transformation of the number of aphids counted on the leaves of Brussels sprouts plants infested with *Brevicoryne brassicae* in relation to different levels of sulfur applied. Each bar is the mean of six values. The vertical line indicates the LSD at $P = 0.05$. Bars that do not share the same letter are significantly different.

the field. For the first four weeks after potting, plants were watered with nutrient mix every two weeks. After that, the nutrient mix was applied weekly, and 500 ml of distilled water was applied whenever supplementary water was necessary.

Aphids were cultured on cabbage (*B. oleracea* var. *capitata*) seedlings and identified according to Blackman and Eastop (1984). The alate form of the aphid, which is the primary dispersive form (Nault and Styer, 1972), was selected for initial infestation.

Just before infestation, six replicates of 10 pots, each replicate consisting of two cultivars of Brussels sprouts grown with five different levels of sulfur, were placed in cages ($1.5 \times 0.75 \times 1$ m) covered by terylene voile. Forty alate aphids were released into each of three cages at two release points, and had a choice of cultivar and sulfur level. The plants in the other three cages were not infested with aphids. Plants that were infested with *B. brassicae* are called infested plants. Plants that were kept healthy and were not infested with aphids are called uninfested plants.

Total apterae aphids were counted on every leaf of the infested plants four weeks after infestation. It was observed that, although there was movement of aphids among plants within the cages, there was a tendency for them to settle and colonize individual plants. This resulted in considerable variation in aphid numbers among plants, and consequently the data were transformed as follows (Steel and Torrie, 1981):

$$\text{Average number of apterae aphids/leaf} = \frac{\sqrt{(\text{total aphid/plant}) + 0.5}}{\sqrt{(\text{total leaves/plant with} \geq 1 \text{ aphid}) + 0.5}}$$

Immediately after the insects were counted, the three most recent fully expanded leaves were harvested from all infested and uninfested plants and assayed for free thiocyanates and total glucosinolates. About 5 g was weighed and oven-dried, and the rest of the material was ground to a fine powder in liquid nitrogen with a mortar and pestle and stored at −80°C until analysis.

The concentration of free thiocyanates was estimated by a modification from Josefsson (1968). Ground tissue (0.5 g) was mixed with 2 ml of water and 0.5 ml of 50% trichloroacetic acid (TCA). To this was added 0.5 ml of 1 M lead acetate and the volume was adjusted to 5 ml with water. The mixture was centrifuged at 4000 rpm for 10 min and the supernatant was filtered through Whatman No. 1 paper. One milliliter of filtrate was mixed with one ml of 0.4 M ferric nitrate and the color allowed to develop for at least 10 min. A blank was made by discharging the color in a duplicate sample with 3 drops of 5% mercuric chloride, and the absorbances were measured at 460 nm. The thiocyanate content in the sample was determined from a standard curve made with a range of concentrations of potassium thiocyanate.

The concentration of total thiocyanates was estimated by incubating 0.5 g of ground tissue in 2 ml of water for 20 min at 22°C to allow myrosinase to degrade glucosinolates. Then 0.5 ml of 50% TCA and 0.5 ml of lead acetate were added and the volume adjusted to 5 ml with water. The mixture was centrifuged and treated as above. No account was taken of a possible effect of sulfur level or cultivar on the activity of myrosinase.

The concentration of total glucosinolates was calculated by subtracting the value for free thiocyanates from that for the estimate of total thiocyanates.

The experiment was designed as a split plot incorporating a randomized complete block design (Steel and Torrie, 1981) with infested and uninfested plants as whole units, and sulfur level and cultivar as subunits. The analysis of variance was carried out using Genstat 5, and the least significant difference (LSD) was calculated at the 0.05 level of probability.

RESULTS

Number of Aphids per Leaf

The mean number of aphids per leaf on infested plants was not significantly affected by the cultivar of Brussels sprouts. When the data for the two cultivars were combined, the mean number of aphids per leaf was affected significantly by the rates of sulfur applied (Figure 1).

Plants that received sulfur at 11.25 ppm had significantly more aphids than all other treatments, except for those that received sulfur at 15 ppm. Plants that received sulfur at 15 ppm had significantly more aphids than those receiving 0 and 7.5 ppm. There was no significant difference in the number of aphids per leaf among plants that received 0, 3.75, or 7.5 ppm sulfur.

Free Thiocyanates

The mean concentration of free thiocyanates in the leaves of cv. Roger, averaged over all treatments, was significantly higher than that for cv. BS 0.85 (41.7 and 37.5 μg/g dry weight, respectively). However, there were no significant interactions of cultivar with either aphid numbers or sulfur levels.

When the data for the two cultivars were combined, a significant interaction was found to occur between aphid infestation and sulfur levels on the concentration of free thiocyanates (Figure 2a). For uninfested plants, there were no significant differences in the free thiocyanate concentration in the leaves of plants at any level of sulfur. For infested plants, the free thiocyanate concentration in the leaves of plants that received 15 ppm of sulfur was significantly higher than for all other levels of sulfur. At 3.75 ppm S, the free thiocyanate concentration of uninfested plants was significantly higher than for infested plants that received 0, 3.75, 7.5, and 11.25 ppm of sulfur, but not significantly different from infested plants that received 15 ppm.

Total Glucosinolates

The mean concentration of total glucosinolates in the leaves of the two cultivars, averaged over all treatments, was not significantly different, and there were no significant interactions of cultivar with either aphid numbers or sulfur levels. When the data for the two cultivars were combined, a significant interaction was found to occur between aphid infestation and soil sulfur levels on the concentration of total glucosinolates (Figure 2b). For uninfested plants, the concentration of total glucosinolates at 15 ppm of sulfur was significantly higher than for all other levels of applied sulfur except for 11.25 ppm. For infested plants, the concentration of total glucosinolates at 15 ppm was significantly lower than that at 3.75 ppm, but not significantly lower than at 0, 7.5, or 11.25 ppm.

The concentration of total glucosinolates in the leaves of infested plants treated with 15 ppm sulfur was lower than that for uninfested plants at all levels of applied sulfur.

DISCUSSION

Reductions in the level of soil sulfur have been associated with reduced amounts of sulfur-containing secondary metabolites in *B. campestris* (Josefsson

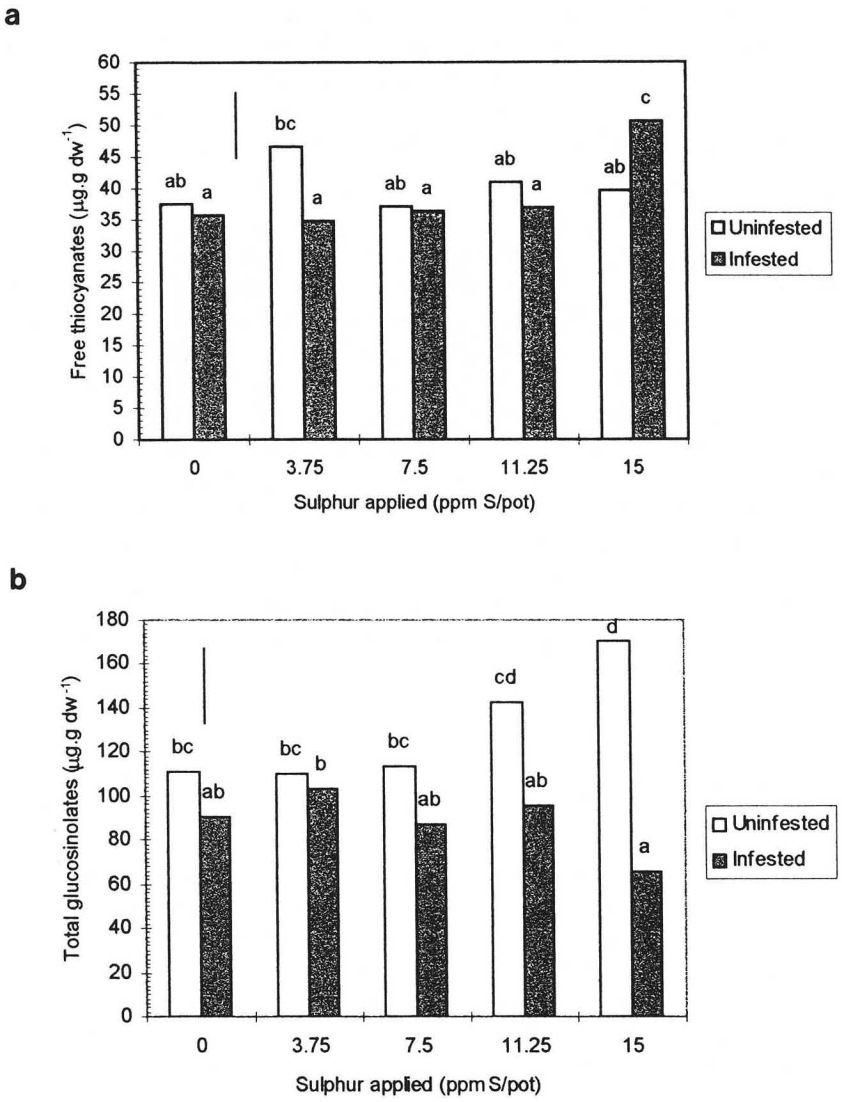

FIG. 2. The concentration of free thiocyanates (a), and total glucosinolates (b), in the leaves of Brussels sprouts uninfested and infested with *Brevicoryne brassicae* in relation to different levels of sulfur applied. Each bar is the mean of six values. The vertical line indicates the LSD at $P = 0.05$. Bars that do not share the same letter are significantly different.

and Appelqvist, 1968), *B. nigra* (Wolfson, 1982), *B. juncea* (Kaur et al., 1990), *B. napus* (Walker and Booth, 1994), and *B. oleracea* (Williams et al., 1996). We found a significant increase in the level of total glucosinolates in the leaves of uninfested Brussels sprouts plants treated with the highest levels of sulfur, which indicates an increase in the pool size of these compounds. This may be attributable to a greater metabolic rate, a decrease in the rate of breakdown, or both.

Van Emden (1990) studied the effect of *B. brassicae* on leaf area, dry matter distribution, and amino acids of Brussels sprouts. He found that the number of leaves, as well as individual leaf area, was reduced and that leaves on infested plants were less efficient assimilators compared to uninfested plants. Infested plants also had smaller root systems and a reduced concentration of leaf amino acids. For the results reported here, the lower overall concentration of total glucosinolates in infested plants compared to uninfested plants could be explained by a reduction in amino acid levels.

Variation between plants in the composition of nutrients and allelochemicals has been suggested to explain patterns of host use by insects (Waterman and Mole, 1989). For example, Cole (1996) found that leaves of *B. napus* plants treated with salicylic acid developed more 2-phenylethyl glucosinolate and supported smaller colonies of *B. brassicae* than control plants.

Our data show that when *B. brassicae* was released onto Brussels sprouts plants growing in pots with different amounts of sulfur, significantly greater numbers of aphids per leaf were found on those plants that received the highest levels of soil sulfur. Since all sulfur treatments were combined within the same voile-covered cage, this indicates a feeding preference by the insects. By contrast, Koritsas and Garsed (1985) found that sulfur had no effect on the preference of aphids for Brussels sprouts.

Van Emden (1990) observed that for plants heavily infested with aphids, the effective leaf area for assimilation was reduced by leaf curling, a phenomenon that was also observed during the period of aphid infestation reported in this paper for young leaves of plants with the highest numbers of aphids.

After one month of infestation, the concentration of total glucosinolates in leaves of infested plants that received the highest level of sulfur and had the greatest number of aphids per leaf was significantly less than for uninfested plants at any level of sulfur. The significant increase in the concentration of free thiocyanates that occurred in infested plants treated with 15 ppm of sulfur indicates that myrosinase activity was stimulated, and, for the same level of applied sulfur, a comparison of the concentrations of free thiocyanates and total glucosinolates shows that glucosinolate metabolism has also been decreased by the presence of the aphids.

We concluded that aphids were initially attracted by the greater concentrations of glucosinolates in those plants that received the highest levels of sulfur. Subsequently the plants responded to the infestation by a depression in gluco-

sinolate synthesis and increased activity of myrosinase, which degraded some glucosinolates into free thiocyanates.

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HOST ATTRACTANTS FOR RED WEEVIL, *Rhynchophorus ferrugineus:* IDENTIFICATION, ELECTROPHYSIOLOGICAL ACTIVITY, AND LABORATORY BIOASSAY

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Abstract—A steam distillate from the freshly cut young bark of coconut palm *Cocos nucifera* was analyzed by gas chromatography, combined gas chromatography–electroantennographic detection (GC-EAD) and GC-MS to detect host attractants for the curculionid weevil *Rhynchophorus ferrugineus*, one of the major coconut pests in Sri Lanka. A twin FID peak consisting of a minor and a major component was shown to possess electrophysiological (EAG) activity. The minor peak was identified as γ-nonanoic lactone **1**, while the major peak was identified as 4-hydroxy-3-methoxystyrene **2**. In an EAG assay the synthetic racemic nonanoic lactone **1** did not elicit a considerable response in the antenna of *R. ferrugineus*, whereas the laboratory synthesized **2** showed activity. In a laboratory bioassay using a Y-type olfactometer, synthetic **1** and **2** elicited moderate attractant properties to *R. ferrugineus*, whereas a 1:1 mixture of the compounds showed increased attraction over that of the individual compounds.

Key Words—Host attractants, *Rhynchophorus ferrugineus*, coconut pest, γ-nonanoic lactone, 4-hydroxy-3-methoxystyrene.

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INTRODUCTION

Rhynchophorus ferrugineus F. (Coleoptera: Curculionidae), commonly known as the red weevil (Wattanapongsiri, 1966; Nirula, 1956) in Sri Lanka, is among five major pests of the coconut palm (Pinto, 1984). The female beetle is known to lay eggs in damaged or wounded tissue of palms. Its larvae feed by burrowing into the fresh tissue, finding their way into the bud region and into the heart of the crown, where they congregate and continue feeding for a period of two to four months. As a result, infested palms die (Rajapaksha and Kanagaratnam, 1988).

Difficulties have been encountered in the detection of infested coconut trees before they reach the stage of complete destruction (CRI, 1975). The present method of control recommended by the Coconut Research Institute in Lunuwila, Sri Lanka, is to drill holes into the soft bud region of the infested palm and to introduce insecticides (e.g., monocrotophos) (Advisory Leaflet No. 37) (CRI, 1976). This practice, however, is not satisfactory because only larvae coming into direct contact with the insecticide are killed and the plant can be saved only when the treatment is performed in an early stage of infestation. Another approach is the use of an electronic device for the detection of red weevils inside the coconut trunk (CRI, 1971). This method is not practical.

The sap oozing from wounded young coconut stem tissue is a widely known attractant for many species of palm weevils, and traps baited with coconut stem tissue have been used to reduce the weevil populations in India (Abraham and Kurian, 1975), the West Indies (Hagley, 1965) and Indonesia (Kalshoven, 1950), while in Sri Lanka they were used only in problem situations (CRI, 1976). Recently, palm tissues have been used to improve the efficiency of aggregation-pheromone-baited traps for *Rhynchophorus* species because the host volatiles were found to be synergistic for the respective aggregation pheromones of the species (Rochat et al., 1993). The introduction of host palm tissues into weevil traps increased the number of weevil catches with the aggregation pheromones of the palmetto weevil, *R. cruentatus* (Weissling et al., 1994), *R. palmarum* (Jaffé et al., 1993), *R. phoenicis* (Gries et al., 1994), and *R. ferrugineus* (Hallet et al., 1993). Traps baited with pheromone from host tissue have also been employed in the management of red ring disease (Griffith, 1987) in commercial oil palm. A reduction of red ring by 80% has been reported by the use of one trap per 5 ha (Chinchilla et al., 1993).

Our preliminary studies have shown that the steam volatiles of the coconut bark are highly attractive to both males and females of *R. ferrugineus* (Gunatilake and Gunawardena, 1986; Gunawardena and Gunatilake, 1993). Isolation of a host attractant for *R. ferrugineus* would allow the food component (coconut stem tissue) of the already used pheromone-food traps to be replaced (Hallet et al., 1993), thus simplifying the trap operations. This is because the species

aggregation pheromone, 4-methyl-5-nonanol (ferrugineol) lasts for more than two months in the field (Gunawardena and Bandarage, 1995), whereas the coconut stem tissue needs weekly replacement. We report for the first time the identification, electrophysiological activity and laboratory bioassay of two host-derived attractants for *R. ferrugineus*.

METHODS AND MATERIALS

Steam Distillation. Young coconut bark (3 kg) up to 10 cm depth from outside of the stem was cut into small pieces and steam distilled for 5 hr in an overall glass apparatus. The distillate (300 ml) was saturated with NaCl and subsequently extracted with diethyl ether (BDH, GPR Grade, 3 × 200 ml). The ether phase was dried over magnesium sulfate and concentrated to 1 ml in vacuo (concentration of **1** and **2** is 0.026 $\mu g/\mu l$ and 0.14 $\mu g/\mu l$, respectively, determined by gas chromatography). Due to the volatile nature of the attractants, the solvent was not completely removed from the extract, and this concentrated etheral solution was used in the laboratory bioassays.

Gas Chromatography. Gas chromatography (GC) was performed on: (1) a Hewlett Packard 5890 A chromatograph equipped with a spitless injector, flame ionization detector (FID), and a fused silica capillary column (25 m × 0.25 mm, SE-30), 4 min at 60°C, 60–260°C at 10°/min, hold, carrier gas N_2; (2) Hewlett Packard 5890 A chromatograph, fused silica column SP-2340 (30 m × 0.25 mm), 4 min at 60°C, 60–195°C at 3°/min, hold, N_2; and (3) Varian 3400 chromatograph, fused silica column SE-52 (25 m × 0.25 mm), 4 min at 60°C, 60–260°C at 6°/min, hold, carrier gas N_2.

Gas Chromatography–Mass Spectrometry (GC-MS). A Varian 3400 gas chromatograph, fitted with a split-splitless injector, coupled to a Finnigan MAT90 double focusing mass spectrometer was used. GC conditions were the same as above in method 3.

Chemicals. Chemical synthesis of 4-hydroxy-3-methoxystyrene **2** was achieved according to Reichstein (1932) by decarboxylation of ferulic acid with quinoline and copper powder. Racemic γ-nonanoic lactone **1** was purchased from Aldrich Chemical Co. Ltd., in Germany.

Electroantennography. Male and female *R. ferrugineus*, 1–5 days old, were air transported from Sri Lanka to Germany, maintained at a photoperiod of 10 hr light and 14 hr dark at 29°C ± 2°C and fed with apples and water. Each weevil (3–8 days old) was anesthetized with CO_2 and the antenna cut off as close as possible to its base. Following the methodology originally described by Schneider (1957), the antenna was fixed on two capillary Ag–AgCl electrodes filled with insect Ringer solution. The recording electrode was inserted into the antennal club on which the olfactory sensilla are located and the indifferent

electrode into the base of the antenna and sealed with vaseline. Olfactory stimuli were applied with an airstream towards the antenna at 3-min intervals passing aliquots on filter paper.

Coupled Gas Chromatography–Electroantennography. Coupled gas chromatography–electroantennographic detector (GC-EAD) (Struble and Arn, 1984) was performed according to GC conditions 2. The column effluent was split between the FID and the antenna and make-up gas (N_2, 20 ml/min) added to the electroantennographic detector (EAD) side to accelerate the GC effluent. The effluent was passed through a heated transfer line into a glass tube (200 mm length, 6 mm ID), the open end of which has been narrowed to 3 mm in order to direct the effluent effectively onto the antenna.

Insects. Larvae of *R. ferrugineus* obtained from infested coconut palms were introduced into a freshly cut young trunk, which was placed in a wooden cage fitted with a wire mesh for ventilation. Adults emerging from the trunk were transferred to a moistened container and fed with sugarcane and 10% sugar solution. Temperature was maintained at 29°C ± 2° and relative humidity at 80 ± 4%. Insects up to the age of two weeks only were used in the bioassays.

Behavioral Bioassay. A choice test was performed in a Y-shaped olfactometer (Gunawardena et al., 1989). The baits were prepared by introducing appropriate amounts of attractants from stock solutions (1 mg/ml ether) on filter paper and allowing the solvent to evaporate. In the case of the natural host attractant, 50 μl of the concentrated ether extract from the steam distillate was used. Blanks were prepared from 50 μl of diethyl ether. Baits were placed in one arm (e.g., A) of the Y tube, and blanks in the other arm (e.g., B), and this sequence was interchanged randomly in the subsequent replicates. A slow stream of air was passed from behind through both arms A and B, and the insect container containing the test insects was fitted into the open end of the third arm C, so that the beetles could move towards A and B against the airstream. After 2 min, the number of insects settled to each arm was counted, and their choice for the baited arm considered as the criterion for activity. Mean numbers of weevils in arms A and B were compared for each bait by the chi-square test. The activities of different baits were compared by ANOVA, and subsequently pairwise comparisons were made with Scheffe's test. The activities of **1**, **2**, and a 1:1 mixture of **1** and **2** were assayed over the dose range 31–750 μg. Subsequently a comparison of the activity of a 1:1 mixture of **1** and **2** (50 μg) was made with the natural host attractant (50 μl) and racemic 4-methyl-5-nonanol (50 μg). Possible host attractant–aggregation pheromone synergism was looked into by separately assaying 1:1 mixtures of the aggregation pheromone (50 μg) and the natural host, 1:2. All bioassays were conducted between 8:00 and 9:00 AM with 10 batches consisting of six weevils in each replicate, as far as possible with equal numbers of males and females.

RESULTS

To search for sensorially active constituents of the palm bark, the steam distillate of young coconut bark was separated by gas chromatography, the column effluent split in a ratio 1:1, and physiologically active compounds recorded with an insect antenna. Thirty-seven minutes after the injection, both female and male *R. ferrugineus* antennae showed a fairly prominent antennal response, the GC-EAD response profiles are depicted in Figure 1a (female) and Figure 1b (male), respectively. In a simultaneously recorded FID chromatogram, the most prominent peak in this elution range was **2** (Figure 1c), and just prior to it an accompanying peak **1** (ratio **1**:**2** = 1:8).

The mass spectrum of **1**, obtained from a subsequent GC-MS analysis, consisted of a base peak of *m/z* 85 and a parent peak *m/z* 138 and was considered to be that of γ-nonanoic lactone. From **2** a spectrum was obtained with parent/base peak of *m/z* 150, congruent with that of 4-hydroxy-3-methoxystyrene (Figure 2). Identical mass spectra as well as retention characteristics on three different GC columns (SE-30, SP-2340, and SE-52, GC conditions given in 1–3) in Methods and Materials) with those of authentic racemic γ-nonanoic lactone **1** and synthetic 4-hydroxy-3-methoxystyrene **2** confirmed the identity of these two palm bark components. Further constituents of the steam distillate were identified as diethylene glycol, triethylene glycol, acetic amide, hexanal, phenyl acetaldehyde, pentylfuran, dihydrobenzofuran and 2-pentanone.

In an electroantennogram (EAG) assay, single 1 sec stimuli of the steam distillate, synthetic **1** in a dose range from 0.1 to 100 μg, **1** as well as the additional identified compounds mentioned above did not elicit considerable electrophysiological responses compared with air controls. Stimuli of **2**, however, showed significant EAG amplitudes and revealed an increase of the electrophysiological activity at higher doses (Figure 3), characteristic for sensorially active compounds. The electrophysiological responses of **2** were even higher than those of equal amounts of the concentrated steam distillate of the natural host attractant.

Investigating the behavioral activity of bark distillate and constituents, the EAG studies were followed by olfactometer test. Searching movements of the snout coupled with rapid movements of the beetles towards the Y junction of the olfactometer and selection of the baited arm within 2 min were considered as positive responses. The results of the behavioral bioassay with the synthetic attractants in a dose range of 31–750 μg are shown in Figure 4. Except in its lowest concentration (31 μg), the γ-lactone **1** always revealed slightly higher activities than the styrene **2**. However, a 1:1-mixture of **1** and **2** attracted significantly higher numbers of red weevils than compounds **1** and **2** individually (e.g., 80.0% vs. 55.6% and 50.0% at 125 μg stimulus concentration). In a final

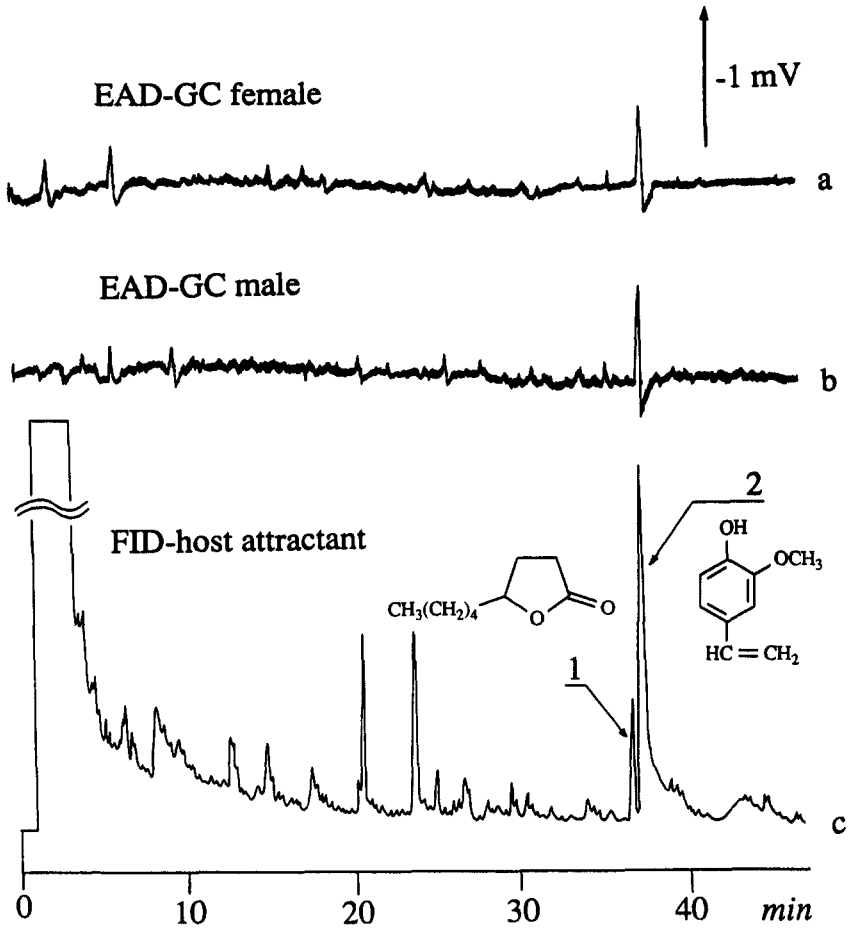
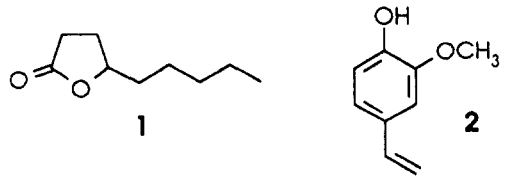


FIG. 1. Electroantennogram detector (EAD) responses of (a) female and (b) male antennae of *Rhynchophorus ferrugineus* and (c) FID chromatogram of a steam distillate of young bark of coconut palm *Cocos nucifera*. **1**, 4-nonalactone; **2**, 4-hydroxy-3-methoxystyrene.



1 2

FIG. 2. Structures of **1** and **2**.

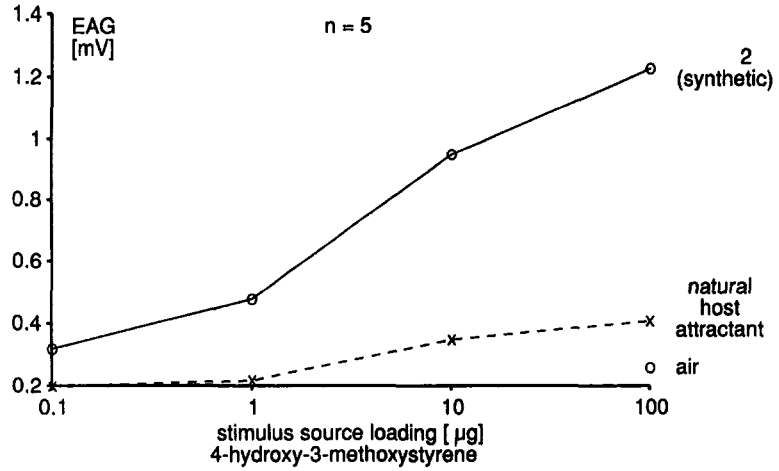


FIG. 3. Dose–response curves of relative electroantennogram response amplitudes of female *R. ferrugineus* to racemic 4-hydroxy-3-methoxystyrene **2** and natural host attractants from coconut bark.

test series, the attractivity of the synthetic mixture of **1** and **2** (50 µg) was compared with that of the concentrated solution of the palm distillate (50 µl) as well as with that of synthetic 4-methyl-5-nonanol (ferrugineol, 50 µg), which is the more attractive component of the aggregation pheromone of the red weevil

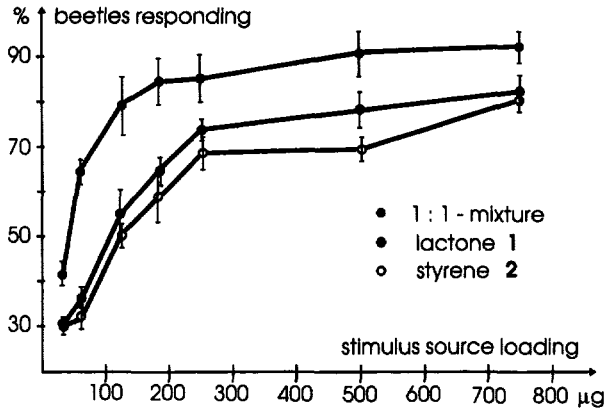


FIG. 4. Mean responses (percentage values) of an olfactometer bioassay with males and females of *R. ferrugineus* to 4-nonanolactone **1**, 4-hydroxy-3-methoxystyrene **2**, and a 1 : 1 mixture of both.

TABLE 1. OLFACTOMETER BIOASSAY RESULTS WITH ADULT *R. ferrugineus*[a]

Attractant (bait)	*N*	Responding (mean ± SEM)		Nonresponding (mean ± SEM)	t_{obs}
		Baited arm (A)	Nonbaited arm (B)	Arm C + insect tube	
Distilled water (50 μl)[b]	10	0.2 ± 0.42c	0.9 ± 0.73	4.9 ± 0.94	2.0
Natural host attractant (50 μl)	10	4.6 ± 0.31a	0.8 ± 0.42	0.6 ± 0.50	19.0
1:1 mixture of **1** and **2** (50 μg)	10	3.4 ± 0.57b	2.1 ± 0.56	0.5 ± 0.48	5.6
Ferrugineol (50 μg)	10	3.7 ± 0.48b	1.7 ± 0.48	0.6 ± 0.52	9.3
1:1 mixture of steam distillate (50 μl) + ferrugineol (50 μg)	10	3.8 ± 0.63b	1.7 ± 0.84	0.6 ± 0.57	6.6
1:1 mixture of **1** + **2** (50 μg) + ferrugineol (50 μg)	10	3.6 ± 0.52b	2.0 ± 0.51	0.4 ± 0.51	5.6

[a]Six insects were used in each experiment. Insects in each arm were counted 2 min after introducing the bait to arm A. Mean number of insects responding followed by similar letters are not significantly different ($P > 0.05$, ANOVA, Scheffe's test).

[b]Except in the case of distilled water, the mean number of insects in baited (A) and nonbaited arms (B) were significantly different ($P < 0.001$, chi-square test).

(Hallet et al., 1993). Distilled water was taken for control comparisons. The 1:1 blend revealed high attractivity similar to that elicited with the species-specific pheromone and also significantly different than the control. No activity increase was observed when aggregation pheromone was mixed 1:1 with the natural or synthetic host attractants (Table 1).

DISCUSSION

The present investigation demonstrates the potency of the 1:1 mixture of synthetic racemic nonanoic lactone **1** and 4-hydroxy-3-methoxystyrene **2** as a lure for the red weevil *Rhynchophorus ferrugineus*. In the choice test, both synthetic **1** and **2** showed moderate attractant properties, whereas the activity of the mixture was significantly higher than those of the single constituents (Figure 4) and almost reached that of the synthetic aggregation pheromone (Table 1). In the case of the control, redistilled water, more than 78% of the insects remained in the C arm of the Y tube and only 3% chose the baited arm. However, because synthetic **1** proved to be essential for the attractivity, it is difficult to understand why it did not evoke much EAG efficacy. A plausible

explanation for this could be that **1** evoked only minute EAG amplitudes that could not be detected under the test conditions used. Furthermore, it is likely that the number of olfactory receptors for **1** is much smaller than the number for **2**. As a consequence, the receptor responses of **1** might be undetectable.

Lactone **1** is chiral and its naturally occurring form could be optically active and exist in one preferred enantiomeric form only. The use of a lure with proper enantiomeric composition might enhance the attractivity of the bait. We plan to synthesize both stereoisomeric antipodes, (*R*)- and (*S*)-4-nonanoic lactone, in optically pure form to elucidate the composition of the naturally occurring attractant by GC on chiral columns.

γ-Substituted butyrolactones are described as defensive secretions from the pygidial gland of staphylinid beetles (Wheeler et al., 1972; Dettner and Schwinger, 1982), but also as a sex pheromone of the female Japanese beetle (Tumlinson et al., 1977), and can be obtained by microbial β-oxidation of suitable oxidized forms of C_{18} fatty acids (Cardillo et al., 1989). 4-Alkyl γ-butyrolactones are ubiquitous natural products and are found in fruits, flowers, tobacco, cooked meat and butter fat (Ravid et al., 1978). Nonanoic lactone **1** is a commercially available artificial coconut-odor component (Abricolin) for apricot and coco fragrances and used as a suntan lotion additive. 4-Hydroxy-3-methoxystyrene **2** is a derivative of ferulic acid (4-hydroxy-3-methoxycinnamic acid) and is formed with various reactions and pyrolytic and heating processes of lignin. Among others, it was found as an aroma component in coffee (Stoll et al., 1967; Gal et al., 1976), tomatoes (Viani et al., 1969), essential oil of Kudzu (Shibata et al., 1978), tar and smoke of hickory (Hruza et al., 1974; Fiddler et al., 1966), and smoke condensates of tobacco (Ishiguro et al., 1976). Generally, it is found in smoked, roasted, heated, and fermented products. More interestingly, alkoxy styrene derivatives were synthesized and tested for attractancy for fruit fly species, and the benzoate of **2** was found to be moderately attractive for male and female melon flies (Shaw et al., 1976).

γ-Nonanoic acid lactone **1** and 4-hydroxy-3-methoxystyrene **2** are new structures as curculionid host attractants, since only simple aromatic compounds such as benzaldehyde, aliphatic alcohols, and terpenes (Dickens, 1990; Müller and Haufe, 1991; Budenberg et al., 1993) are known so far as attractants for this coleopteran family. 3-Methylindol has been used as an artificial attractant in field trapping of *R. palmarum* rather successfully in the West Indies, although it was not confirmed as a host attractant (Hagley, 1965). Recently several simple and host-derived alcohols, acetates, aldehydes, and esters of carbon chain lengths varying from C_2 to C_6 were recognized as synergists to the respective aggregation pheromone of *Rhynchophorus* species in the field. For example, some synergists for the aggregation pheromones of three palm weevils are as follows: ethyl acetate or a mixture of ethyl alcohol, ethyl acetate, pentane, hexanal, isoamyl acetate, or isopentanol for *R. palmarum* (Jaffé et al., 1993); ethyl

propionate, ethyl butyrate, and ethyl isobutyrate for *R. phoenicis* (Gries et al., 1994), and ethyl acetate, ethyl lactate, ethyl butyrate, ethyl isobutyrate, and ethanol for *R. cruentatus* (Giblin-Davis et al., 1994). However, these compounds alone did not attract the weevils in the field. This led to the assumption that in these field attractant lures, viz. aggregation pheromone-host derived small molecules, the former acts as a long-range attractant, while the latter provides the short-range orientation cues to the bait (Jaffé et al., 1993; Weissling et al., 1994). The fact that palm tissues attract weevils from a distance in the absence of species's aggregation pheromone suggests that the host palms produce their own long-range attractants in addition to short-range attractants recognized above. The field observation that weevil catches for the host tissue–aggregation pheromone combination have often been higher than those of the host derived small molecule aggregation pheromone (Giblin-Davis et al., 1994; Gries et al., 1994) adds proof to this. The two host attractants **1** and **2** do not conform to the structural characteristics of short-range attractants known so far in the family Curculionidae in that they are of higher molecular weight, asymmetric, and one of them is chiral. Compounds **1** and **2** did not show synergism with the species aggregation pheromone, ferrugineol (Table 1). It is therefore likely that **1** and **2** are the host's long-range attractants. This, however, should be proved by a field bioassay. Literature evidence of long range attractants is rather scant in the family Curculionidae.

Young coconut palms are known to be more susceptible to red weevil attack than older ones. Since lignin as the possible precursor of **2** is found in many plants, however, it might be the physiological state of the young plant determines the content of the 4-hydroxy-3-methoxystyrene **2**. It is furthermore unlikely that the methoxystyrene alone is responsible for the host attraction. The synergism between the methoxystyrene and the lactone observed in the bioassay adds proof for this. Although the lactone **1** was shown to be electrophysiologically inactive compared with the methoxystyrene **2**, the responses by weevils in the behavioral assay appeared stronger to the former than to the latter (Figure 4). This suggests the possibility that the lactone **1** is an even more essential ingredient for the red weevil's attraction to the host volatiles. The possibility that there are still other important attractant components in the host volatiles can not be ruled out. Several coconut stem tissue-derived attractants already identified, i.e., ethanol, ethyl acetate, pentane, hexanal, isopentanol, and amyl acetate by headspace analysis (Jaffé et al., 1993) were not found in our steam distillates. In effect, our method of isolation, i.e., steam distillation, has yielded volatile attractants with higher molecular weight.

Identification of **1** and **2** in the host volatiles of the coconut bark is perhaps the first major breakthrough in understanding the host finding behavior of *R. ferrugineus*. This knowledge, coupled with the recently isolated aggregation pheromones of the species will lead to an efficient lure for the red weevil whose

detection has been difficult in the past. The fact that host attractants **1** and **2** are readily available compounds would make their use more economical and practical. Traps baited with **1** and **2** with and without ferrugineol would indicate whether they possess long-range attractant properties or they act more like short-range orientation cues for the weevils. Depending on the results, **1** and **2** may be combined with the species aggregation pheromone or potent short-range attractants in order to formulate an efficient lure for the red weevil.

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LABORATORY EVALUATION OF ODOR PREFERENCES OF THE BRUSHTAIL POSSUM

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Abstract—The Australian brushtail possum (*Trichosurus vulpecula*) is the major vertebrate pest in New Zealand. Possums cause significant damage to the country's native and exotic forests and, as a vector of bovine tuberculosis, are a serious threat to the country's meat industry. Strong smelling odors are often used as lures during possum control operations in New Zealand, but little is known about the preferences of possums for these odurs. A test was designed to determine the preferences of captive wild possums for a number of odors commonly used as lures. To assess the effectiveness of the method, the possums were tested for their responses to a familiar food odor (apple) versus no odor. The possums spent significantly more time investigating the apple odor and also sniffed it more often than the no-odor control in the 15-min test period. The same procedure, however, showed that five odors commonly used as lures (almond, cloves, cinnamon, peanut, and orange) were no more preferable than water. Our results suggest that these unfamiliar odors are either equally attractive or unattractive to possums and that odors that are more familiar to these animals may be more appropriate as lures.

Key Words—Brushtail possums, *Trichosurus vulpecula*, lures, odor preferences, pests, behavior, familiarity.

INTRODUCTION

The Australian brushtail possum, *Trichosurus vulpecula* (Kerr), was introduced into New Zealand in the mid- to late 19th century to establish a fur trade (Pracy,

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1974). By the 1940s, possums in New Zealand had eliminated up to 70% of the trees and shrubs in some native forest areas (Batchelor and Cowan, 1988). In 1967, bovine tuberculosis was identified in possums (Ekdahl et al., 1970) and subsequently the possum was identified as an important vector of the disease (Livingstone, 1991). At present, 60–70 million possums occupy more than 90% of the country (Batchelor and Cowan, 1988). The threat to New Zealand's native and exotic forests (Batchelor and Cowan, 1988) and the threat to the meat export industry from tuberculosis has led to the designation of the possum as a pest under the Noxious Animals Act of 1956 (Crawley, 1973).

A variety of odorous lures have been used in an attempt to improve the efficiency of the poison baits and traps used during control operations (Morgan, 1990; Gilmore, 1967). Odor cues are considered to be the most likely to attract possums since these solitary, nocturnal marsupials are presumed to be highly dependent on their olfactory sense.

Brushtail possums have a large olfactory bulb, well-developed vomeronasal region, and many scent glands (Russell, 1987). Most of the lures involved in the control of brushtail possums are derived from foodstuffs such as artificial spices and flavorings in the form of oils. There has been some investigation into the effectiveness of these odors as lures. Cowan (1987) compared the success of plain flour as a bait for traps with the success of flour lured with aniseed oil. The lured flour was found to improve the recapture rate of possums in their mark–recapture study, but it did not improve the initial capture rate. Recently, Morgan et al. (1995) evaluated the attractiveness of 20 different odorous compounds. They measured the searching and sniffing responses of possums in pens and found that five of the odors were more attractive than the water control. However, these odors (cinnamon, aniseed, cherry, orange, and plum) were not found to mask the hydrocyanic gas released from the cyanide, and only cinnamon increased the kills achieved with cyanide in the field. Cinnamon was also tested as a lure for bait stations, but did not increase the number of possums visiting the lured bait stations. Thus, although some odors were initially found to be more attractive to possums when they were all offered together in a pen test, these were not successful masks or lures in the field.

It is possible that these lures are inappropriate because the possums do not prefer the odors. We suggest that it is more appropriate to identify first those odors that are preferred by possums and then to test these odors for their effectiveness as lures. Preference may be defined as the relative choice made by an animal following sensory appraisal of the alternatives (Matthews, 1997). In this study, a sniffing preference test was designed and assessed for its ability to determine the odor preferences of possums. Although previous studies have found sniffing tests to be appropriate for determining odor preferences in other species (Johnston, 1977), this has not been evaluated for brushtail possums. The test was then used to determine the preferences of possums for five food

odors, each of which are currently used in combination with traps and poisoned baits in the field.

METHODS AND MATERIALS

For each experiment, 12 possums were captured from the bush–pasture margin at the Whatawhata Research Station near Hamilton, New Zealand, and transported to quarantine facilities for six weeks before being transferred to cage facilities at the Animal Behaviour and Welfare Research Centre (ABWRC), Ruakura, Hamilton. The possums ranged in age from 1 to 9 years old in Experiment 1, and 1 to 4 years old in experiment 2, as determined by tooth wear (Kean, 1975).

The possums were housed in a single room in individual wire cages (640 × 500 × 730 mm high), each with a shelf (400 mm from the base), a nestbox (400 × 250 × 250 mm high), and a water nipple. The light conditions (12L : 12D) were kept in a reverse cycle (dark from 10 : 30 to 22 : 30 hr) to allow the nocturnal behavior of the possums to be observed during work hours. Animals were exposed to natural temperature variation (10–30°C) and fluorescent lights were used to simulate daytime light levels.

The diet consisted of a wet mash composed of Northern Rolling Mill (NRM, New Zealand Ltd.) pellets mixed with water at a ratio of 1 : 1.5. Over a three-week pre-experimental period, the 12 possums were trained to eat mash for half an hour immediately following the start of the dark period. The mash was presented in plastic trays that were placed into one of two steel holders attached to the front wall of the cages (each 50 mm from the left or right wall of the cage). The side of mash presentation (i.e., left or right) was varied on an eight-day, single paradiddle schedule (L, R, L, L, R, L, R, R), so that the mash did not appear on the same side for more than two days in succession. Each possum was also fed an apple, placed in the bottom of the cage, in the middle of the dark period.

During the tests, the cages were illuminated from above by a 60-W red light bulb. Two extractor fans (one at each end of the room) expelled air from the cage room during the tests to prevent build-up of test odors during the study by drawing the odors through the subjects' cages.

Odors were presented in perforated plastic specimen jars. During the three-week pre-experimental period, two of these jars were attached to the front of each cage to allow the animals to become accustomed to their presence. Each jar was placed halfway up the front of the cage, and the two were separated by the width of the cage (approximately 600 mm). This placement of the odor jars required the possums to make an obvious effort to sniff the odors either from a position on the floor of the cage or from the shelf. Prior to the start of the preference test, subjects were observed for their reactions to the apparatus.

During the preference tests, two test jars replaced those put on the cage during the three-week pre-experimental period. After each test, the odor jars were removed, and empty specimen jars put back in their place.

The 12 possums in each experiment were observed in four groups of three; six were observed on one day, and six the next. The two groups of three observed each day were alternated between the first and second observation periods over successive days. Groups were observed for 15 min, with observations of one group starting at the beginning of the dark period and observations of the second group starting 30 min later. The test odors were placed on the cages at the beginning of each 15-min test period. The observer had no difficulty observing the responses of the three possums in each test period.

The possums were housed in a two-tier cage bank that was six cages long. This allowed for the three animals in each group to be located in cages that were diagonally separated from each other. Thus, two of the three possums observed were on one level (separated by a single cage) and the third possum was located on the other level. This arrangement ensured that the responses of each possum were to the odors on its own cage, rather than those on its neighbours'. Arranged in this manner, the three possums in each test were unable to see each other.

By observing the possums at the normal feeding time, we ensured that possums were hungry and therefore motivated to respond to the odours. The six animals observed each day were fed immediately after the observation period. All other possums were fed at the start of the dark period, as occurred during the three-week pre-experimental period.

Behavioural Measurements. Sniffing was recorded whenever a possum's nose was within 3 cm of the odor jar. The grid of wires on each cage marked this distance on a two-dimensional plane around each odor jar, and the 3-cm distance into the cage was estimated by eye. Continuous samples were recorded with "Keybehaviour," a behavioural data-collection program (Deag, 1993), on a Hewlett Packard 95LX palm top computer. All observations were performed by one person. The following data were collected and analysed: (1) latency: time until each odor was first sniffed; (2) frequency: total number of times each odor was sniffed in the 15-min observation period; and (3) duration: total amount of time spent sniffing each odor in the 15-min observation period.

During the tests, some animals did not sniff both odor jars. While this did not affect the mean frequency and duration data, it did effect the latency data. This problem has been encountered previously in behavioral studies (Theobald and Goupillot, 1990; Stewart et al., 1980). For the latency data, a notional time of 16 min was ascribed to those individuals that did not sniff an odor.

Experiment 1: Evaluation of Preference Test. The seven female and five male possums that served as subjects in this study were fed apples daily. These were consumed readily, so one might expect the odor of apples to be attractive

to the subjects. In this experiment, apple odor was paired with "no odor" to determine whether a sniffing test was appropriate for assessing odor preferences in the possum.

Before each observation period, slices of apple were placed into a jar for each animal. To prevent the animals receiving any visual stimuli from the apple, all the odor jars (including those containing no odor) were covered on the inside with white paper. Holes were punched through the paper to allow the apple odor to escape through the perforations.

The preference test was conducted twice for each animal. On the second test, the side of presentation of the apple and no-odor jars was changed to counterbalance any side biases in the test animals. The position of the apple jar in the first test was selected randomly for each animal.

The data were analyzed using the ANOVA procedure in the Genstat statistical package (Genstat 5 Committee, 1993). The data were transformed to stabilize the variance and make the data residuals more normally distributed; the duration data were log transformed, a square-root transformation was made on the latency data, and frequency data were increased by one and a square-root transformation was performed. Systematic effects considered were odor, side of presentation, and first versus second test and their first-order interactions. Random block effects were possum, possum × test, possum × side, and possum × odor. The mean duration and latency data from the final model were back-transformed to produce means and SEs. For the frequency data, estimates of the means were obtained by fitting the final model as a Poisson response using the GLMM (generalized linear mixed models) procedure (Breslow and Clayton, 1993) in Genstat.

Experiment 2: Evaluation of Odor Preferences. Six female possums and six male possums served as subjects. The five essential oils used as odors in this trial were orange, cinnamon, peanut, cloves, and almond. These were obtained from Bush-Boake Allen (Auckland, New Zealand) and consisted of highly concentrated pure extracts consistent with regulation 87 (the general standards for edible fats and edible oils) of the New Zealand Food Regulation Act (1984). Distilled water was used as the odorless control. Each possum had its own set of six test jars: one for each odor and the control.

All 15 pairwise combinations [i.e., $n(n - 1)/2$; $n = 6$) of these six stimuli were tested. Each pair was presented only once to each possum. Four drops (approximately 15 μl) of one of the six solutions was placed onto a piece of filter paper (six qualitative, 4.25 cm in diameter), which was then placed into a perforated plastic specimen jar. The filter paper was suspended so that it did not come in contact with the plastic jar. Any stain left on the filter paper by the odor was matched by a similar stain left by the water control and consequently the insides of the odor jars were not covered by a paper shell as they were in experiment 1.

Each of the three possums observed during a given test was always presented with the same two odors. This restricted the number of odors present in the cage room environment at any one time. The side on which each odor was presented was alternated between the three animals and each odor pairing was given in a random order for each group of three animals.

The data were analyzed by fitting models to test for the effect of systematic and random sources of variation using the REML (residual maximum likelihood) procedure (Patterson and Thompson, 1974) in the Genstat statistical package. The data were transformed to stabilize the variance and make the data residuals more normally distributed; we took the square root of the latency data, the duration data were log transformed, and the frequency data were increased by one and a square-root transformation was performed. The main systematic effects considered were odor, side of presentation, and observation period. We also tested whether the responses varied with the number of times an odor had been previously encountered and the number of times a possum had been previously tested. First-order interactions of these systematic effects were also tested. The random sources of variation considered were those due to day, possum, possum $\times$ side and possum $\times$ day interactions. Likelihood ratio tests (asymptotically χ^2) were used to determine which systematic and random effects were retained in the final model. Again, the mean duration and latency data from the final model were back-transformed to produce means and SEs, and estimates of the mean frequencies were obtained by fitting the final model as a Poisson response using the GLMM procedure in Genstat.

RESULTS

Experiment 1. Observations made at the end of the three-week pre-experimental period when no odors were present showed that the possums were responding to the test apparatus, but the level of the response was low. On average, the possums investigated the empty odor jars 1.50 times during the 15-min observation period, with a latency of 635 sec and a duration of 17.4 sec. Two subjects were excluded from the analysis of the preference test. The first failed to approach either jar during the preference tests. The other did not have both choices available during the trial as it removed one of the jars part way into the test.

The results of the ANOVA analysis showed that the apple odor was investigated more often in the 15-min period than the no-odor stimulus ($P < 0.001$). The apple odor was also investigated for a longer time than the no-odor stimulus ($P < 0.001$). The analysis also suggested the apple odor was investigated first, but this was not significant ($P = 0.13$). The analysis found that none of the

TABLE 1. BACK-TRANSFORMED MEANS AND SEs FOR FREQUENCY, DURATION, AND LATENCY DATA FROM EXPERIMENT 1

	Mean frequency (per 15 min)	Mean duration (sec)	Mean latency (sec)
Apple odor	2.01	50.6	386
No odor	1.02	16.0	601
SE	0.39	6.20	130

other systematic or random effects were having a significant affect on the data. Table 1 gives the back-transformed means and SEs resulting from the analysis.

Experiment 2. The analysis identified the important sources of variation. There was no average bias to the left or right, but possum × side variation was an important random effect for all three behaviors measured; that is, some possums tended to be biased towards the left side and some to the right side. The frequency and duration data were also influenced by possum and possum × day effects; some possums spent more or less time sniffing than others and this effect was not consistent from day to day.

Analysis of the frequency and latency data revealed no significant differences between the responses shown to each of the odors. There was, however, a significant difference in the amount of time spent investigating the odors ($\chi^2 = 16.8$, 5 *df*, $P < 0.01$). The analysis showed that on average the possums spent more time investigating the clove odor than orange, almond, and peanut odor, with peanut being investigated for the least amount of time. There were 104 occasions when the possums did not investigate an odor (i.e., 104 zero responses). When these were removed from the analysis the difference in the amount of time spent investigating the odors was not significant ($\chi^2 = 9.76$, 5 *df*, $P < 0.10$). This was probably due to the larger number of zero responses for the orange, almond, and peanut odors than for the clove odor. There were no significant differences, however, in the responses shown to the five odors and those to the water stimulus. Table 2 gives the back-transformed means and SEs resulting from the analysis.

The responses to the five odors and water were also not significantly different from the responses shown prior to the start of the trial. The responses to the empty odor jars before the start of the trial were: mean duration = 33.2 sec, mean latency = 436 sec, and mean frequency = 2.10/15 min.

There was however, a significant effect of observation period. Odors were investigated for a longer time ($\chi^2 = 12.9$, 1 *df*, $P < 0.001$) and more quickly

TABLE 2. BACK-TRANSFORMED MEANS AND SEs FOR DURATION, LATENCY, AND FREQUENCY DATA FROM EXPERIMENT 2

	Mean duration (sec)	Mean duration (sec) with zero responses removed	Mean latency (sec)	Mean frequency (per 15 min)
Peanut	17.7	37.3	430	1.84
Almond	22.6	27.3	406	2.00
Orange	23.1	32.0	417	2.22
Cinnamon	30.0	39.2	409	1.79
Cloves	53.3	56.2	378	2.15
Water	32.5	40.9	391	2.16
SE	8.79	10.1	57.0	0.27
range	6.30–12.8	7.90–13.0	53.8–61.8	0.24–0.30

($\chi^2 = 12.1$, 1 *df*, $P < 0.001$) in the second observation period conducted on each test day.

DISCUSSION

The results show that it is possible to measure the odor preferences of possums using an olfactory sniffing test. The possums showed a distinct preference for the familiar apple odor used in experiment 1. However, the possums' responses to the five unfamiliar odors used in experiment 2 were not significantly different from their responses to the water stimuli. This suggests that these odors were not highly preferred by the possums.

Although there was a suggestion that the possums spent more time investigating the clove odor than the orange, almond, and peanut odors, this was strongly influenced by the large number of zero responses. When the zero responses were removed from the analysis, the difference was no longer significant since the average time spent investigating the orange, almond, and peanut odors increased. This suggests that the difference was mainly due to the possums displaying less interest in the orange, almond, and peanut odors, rather than the possums being more interested in the clove odor. Furthermore, the responses shown to each of the odors were not significantly different from those shown towards water. These responses suggest that the possums either have an equal preference or no preference at all for the five test odors.

An increase in sniffing activity was observed during the second 15-min observation period of experiment 2. This may have been related to an increase in the level of hunger of the possums at this later time. However, since each

individual possum and each odor was balanced for observation period, this did not bias the measurements obtained.

A large degree of intersubject variability was observed in experiment 2. In situations where stimuli are similarly preferred, a large amount of individual variation would be expected. For example, in a study of Matthews (1983), cattle feeds that had a similar preference value were ranked differently by different cows. On the other hand, feeds that differed the most in preference value were usually ranked similarly on a scale of preference. Shumake et al. (1973) suggested that the intersubject variability shown in their test of odor preferences in rats may have been too great to allow adequate tests of preference with weak attractants and small numbers of animals. This may also have been the case in the present study, since the number of animals was small and the stimuli apparently not highly attractive.

The lack of preference for the five test odors may have been a result of the possums inability to discriminate between the odors. Alternatively, the animals may simply not have been motivated to choose among the odors, or the odors may have been equally preferred (Brown, 1979). This is a common problem with preference tests in which no preference for one substance over another is shown (e.g., Clapperton et al., 1988; Winberg and Olsen, 1992; Holmes, 1992). Operant tasks or discrimination tasks [such as that performed by Walker and Croft (1990)] would determine whether brushtail possums are able to discriminate between odors. Such tasks would also provide more information on the sensory abilities of possums, a matter about which little is known.

The results of experiment 2 are consistent with the findings of studies into the effectiveness of these odors as lures (e.g., Morgan et al., 1995). Although Morgan et al. (1995) found some of the odors in their initial pen test to be significantly more attractive than the water control, their field tests showed these odors were not effective as lures. The results of this study suggest that this may be due to the low preference shown by possums for these odors.

The possums, however, did show a significant preference for the apple odor. It is possible that the use of an actual piece of apple inside the odor jar may have provided the possums with odorous cues not given by the five essences. An investigation into the responses of possums to whole foods and to essences may reveal differences that would indicate whether the test odors would be attractive if presented as whole foods. However, most lures used in possum control are currently based on essences.

Familiarity with a substance is considered very important for forming preferences in animals (Rozin, 1976; Shumake, 1978). Animals may show preferences for familiar food odors (e.g., Valsecchi et al., 1993), sexual or conspecific odors (e.g., Halpin, 1978; Stern, 1970), or even odors with no social significance (e.g., Porter and Etscorn, 1976). The possums that served as subjects in experiment 1 were familiar with the apple odor, having eaten apples prior to

the start of the experiment. The possums in experiment 2, however, had never experienced the test odors, and this may explain the lack of preference observed for these odors. If familiarity is important for forming preferences in possums, it may be possible to give possums experience with an odor through the use of a prebaiting strategy. Further experiments could involve the placement of nontoxic baits impregnated with an odor into the area to be controlled. Under these circumstances, possums that ate the nontoxic baits might become familiar with the odor, possibly developing a preference for the odor through this positive ingestive experience. This odor might then be used successfully as a lure.

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QUANTITATIVE VARIATION IN CHEMICAL DEFENSE WITHIN AND AMONG SUBGENERA OF *Cicindela*

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Abstract—The pygidial secretions of more than 40 species of adult *Cicindela* were quantitatively assayed by gas chromatography and mass spectrometry for the presence of benzaldehyde, the major defensive compound of cicindelids. All species showed evidence of benzaldehyde secretion, with the range in benzaldehyde spanning three orders of magnitude. Subgenera, species, and subspecies all accounted for a significant portion of the total variation in benzaldehyde secretion, while populations within subspecies sampled at different localities did not vary significantly; thus phylogenetic analyses of benzaldehyde secretion within and among both species and subgenera would be appropriate. The evidence suggests that a single origin of the pathway producing benzaldehyde occurred in the ancestors of *Cicindela* spp. and that differences among taxa in the amount of benzaldehyde secreted are the result of changes in that pathway's regulation.

Key Words—Insecta, Coleoptera, Cicindelidae, *Cicindela*, benzaldehyde, evolution of chemical defense, lineage variation, predator deterrence.

INTRODUCTION

Members of the family Cicindelidae (tiger beetles) are unique among ground-dwelling beetles for producing benzaldehyde in the pygidial gland (Moore and Brown, 1971; Blum, 1981). Tests of many beetles in the suborder Adaphaga,

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of which Cicindelidae is a member, have revealed that benzaldehyde is not found in the pygidial secretions of any other lineage (Blum et al., 1981). Other arthropods that produce benzaldehyde include millipedes (Conner et al., 1977; Duffey et al., 1974), centipedes (Jones et al., 1976), larval chrysomelid beetles (Moore, 1967), larval sawflies (Duffield et al., 1990), harvester ants (Blum et al., 1969), butterflies (Brower, 1984; Jacquin et al., 1991), and locusts (Torto et al., 1994).

The pathway of benzaldehyde production by millipedes has been determined: mandelonitrile is produced through a number of steps from the precursor phenylalanine and then modified to yield benzaldehyde and hydrogen cyanide (Towers et al., 1972; Duffey et al., 1974; Duffey, 1981). It is likely that the production of benzaldehyde by tiger beetles is similar to that of millipedes (Pearson et al., 1988) because mandelonitrile, hydrogen cyanide, and benzaldehyde all have been detected in the pygidial reservoir of *Megacephala* (Blum et al., 1981; Davis and Nahrstedt, 1985). However, the experiments necessary to verify the pathway in cicindelids have not been conducted. There is no evidence that the compounds of cicindelid pygidial secretions are sequestered plant or prey toxins; therefore benzaldehyde is assumed to be produced *de novo* by tiger beetles (Blum, 1981; Duffey, 1981).

The function of cicindelid pygidial secretions is assumed to be defense against predators. Benzaldehyde is an aromatic aldehyde having a bitter almond smell. It has a low molecular weight (106.13), a large diffusion coefficient (Budavari, 1989), and is easily detected by olfaction. Like other aldehydes, it is probably a nonspecific irritant (Pasteels et al., 1983) that deters invertebrate predators by blocking their antennal chemoreceptors (Duffey et al., 1977; Duffey, 1977). Thus, it may not be as effective a deterrent to vertebrate predators, including insectivorous birds (Pasteels et al., 1983; Brower, 1984; Pearson, 1985; but see Duffey et al., 1977; Clark and Shah, 1994). In contrast, hydrogen cyanide is toxic to both invertebrate (Davis and Nahrstedt, 1985; Peterson, 1986) and vertebrate (Davis and Nahrstedt, 1985) predators. Thus, benzaldehyde may be an effective aposematic signal of hydrogen cyanide to some kinds of predators.

The importance of benzaldehyde production in tiger beetle ecology has been highlighted by studies that suggest the compound deters some predators (Pearson, 1985) and is associated with the habitat in which a given tiger beetle taxon is found (Pearson et al., 1988; Altaba, 1991). If the habitats of beetle taxa reflect the kinds of predators to which they are exposed, then the association between the presence of benzaldehyde and the habitats of taxa may indicate selection by predators on the production of benzaldehyde in cicindelids (Pearson et al., 1988). Additional evidence, however, suggests that the distribution of benzaldehyde production among taxa is associated with the phylogenetic history of lineages (Pearson et al., 1988; Mooi et al., 1989). Because there has been

considerable debate regarding the evolution of benzaldehyde production in tiger beetles (Vogler and Kelley, 1996), new data were required to evaluate the arguments better.

The goals of this study were: (1) to quantify secretion of benzaldehyde in representative species and subspecies of *Cicindela*, (2) to assess variation in benzaldehyde secretion among different taxonomic groupings, and (3) to estimate the amount of benzaldehyde secreted by individual beetles. Quantification was necessary to conduct more precise tests of hypotheses for the evolution of benzaldehyde production. All previous studies provided either no estimate (Moore and Brown, 1971; Blum et al., 1981; Hefetz et al., 1984) or only relative estimates (Pearson et al., 1988) of benzaldehyde production in cicindelids. Statistical power was gained by examining samples of many individuals from populations representing different taxonomic groupings (subgenera, species, subspecies and localities) of cicindelids. The study focused on benzaldehyde as opposed to other pygidial secretions, because mandelonitrile is very unstable, hydrogen cyanide cannot be readily detected from secretions collected in the field, and other compounds have not yet been identified (Blum et al., 1981; Pearson et al., 1988).

METHODS AND MATERIALS

Insects. Beetles were collected either by handnet during the day or by hand at a blacklight during the night. As beetles and their secretions were collected, observations of behavior related to the secretion of pygidial compounds were noted. For each taxon at a given locality, beetles were collected and pinned for identification. All beetles used in the assay of secretions were identified (by K.C.K.) according to the classification scheme of Rivalier (1954, 1961, 1963), as summarized by Boyd and Associates (1982), and saved as voucher specimens. Specimens are deposited in The Field Museum (Department of Zoology, Division of Insects), Chicago, Illinois.

Collection of Secretions. The secretions of beetles were collected by placing a single individual into a 20-ml bottle containing 15 ml of pesticide-grade methylene chloride; this caused the beetle to secrete the contents of its pygidial gland into the solvent (Pearson et al., 1988). No more than one beetle was ever placed into a sample bottle, nor were solutions from individuals combined at any stage of analysis (see *Chemical Analyses* below). To be included in the analysis, the beetle had to be captured after only a short pursuit and placed in the bottle within 10 sec of netting or hand-capturing. Beetles were removed from the solution within 48 hr and stored in ethyl alcohol. In the field, solutions were stored in coolers ($<21°C$); in the laboratory, solutions were stored in a refrigerator ($0 \pm 5°C$). Note that benzaldehyde "secretion" refers to that amount of benzal-

dehyde that was secreted by the individual at the time of capture and before removal of the beetle from the bottle; therefore it may be a combination of the amount of compound stored in the pygidial gland and produced instantaneously at the time of capture.

The target sample size of secretions from 15 individuals for each taxonomic unit was met or exceeded for more than 70% of the taxa (38 of 53; Table 1). The harmonic mean (which tends to exaggerate the small values in a data set and is always smaller than the arithmetic mean) of all taxon sample sizes was 11.

Chemical Analyses. For all analyses, a 100-μl sample of each solution was pipetted into an autosampler vial fitted with an insert. To reduce carryover of benzaldehyde between sampled solutions, the pipet was rinsed with methylene chloride three times between samples. After approximately 15 samples had been pipetted, a 100-μl sample of methylene chloride was pipetted to assess the amount of carryover. This measurement of carryover was important to evaluate accurately samples with very low concentrations of benzaldehyde. Once the vials were prepared, they were used immediately or refrigerated until use within 48 hr. To minimize evaporation during analyses, vials were placed in an autosampler tray and cooled to 8.0 $\pm$ 0.1°C with a Lauda Brinkmann Super RM6 water bath.

The qualitative and quantitative analysis of benzaldehyde was performed in a high-resolution gas chromatography–quadrupole mass spectrometry system. Gas chromatography (GC) was performed on a Hewlett Packard (HP) 5890 Series II Plus gas chromatograph. A cross-linked 5% phenol–methyl–silicone capillary column (HP 5MS; 30 m $\times$ 0.25 mm ID, 0.25-μm film thickness; helium carrier gas 36.8 cm/sec) was temperature programmed (50–200°C at 10°C/min) to enable the separation and identification of benzaldehyde. Injection and detection temperatures were set at 275°C and 300°C, respectively.

For the quantitative analysis of benzaldehyde, the GC system described above was coupled to an HP 5972 Series mass selective detector operated under electron impact mode. Spectra (at 70 eV electron energy) were acquired from 50–600 amu (1.5 scan/sec), and benzaldehyde was unambiguously identified by comparison of the resulting spectrum and retention time (5.81 min) with those produced by authentic standards. Selected ion monitoring (SIM) mode was employed with the ions arising from benzaldehyde (m/z = 106, 105, 77) specified. Dwell times for these ions were adjusted to ensure a minimum 10–15 samples taken across the benzaldehyde peak to ensure reproducibility. The electron multiplier voltage was adjusted to produce maximum signal to noise on a 0.1-ng standard. Samples were assayed first using a 25:1 split ratio. If no benzaldehyde was detected, the sample was reassayed using a splitless injection. The detection limit was approximately 0.01 ng.

TABLE 1. CICINDELID TAXA SAMPLED FOR BENZALDEHYDE[a]

Taxon	Subgenus	Locality	Date	*N*	Benzaldehyde (ng/beetle, mean ± SD)	Carryover	Not zero	Normal	Pearson et al.
C. abdominalis	*Cicindelidia*	NC, Richmond Co., Marston, North Carolina Raceway	May 14, 1995	16	4,698 ± 4,727	0	Y	Y	+
C. auraria euryscopa	*Habroscelimorpha*	Mexico, Sinaloa, Altata	July 19, 1995	10	328 ± 206	0	Y	Y	n/a
C. blanda	*Ellipsoptera*	FL, Santa Rosa Co., Milton, Blackwater River SP	June 14, 1994	17	3,785 ± 2,507	0	Y	N	n/a
C. californica brevihamata	*Habroscelimorpha*	Mexico, Sinaloa, Barra de la Piaxtla	July 18, 1995	6	8,354 ± 13,730	0	N	N	n/a
C. californica brevihamata	*Habroscelimorpha*	Mexico, Sonora, Empalme	July 22, 1995	4	2,511 ± 1,596	0	N	Y	n/a
C. californica mojavi	*Habroscelimorpha*	CA, Imperial Co., Westmorland, Salton Sea NWR	May 8, 1995	16	1,453 ± 1,301	0	Y	Y	n/a
C. californica pseudoerronea	*Habroscelimorpha*	CA, Inyo Co., Beatty Junction, Death Valley NP	May 5, 1995	17	1,254 ± 1,183	0	Y	Y	n/a
C. carthagena carthagena	*Cicindelidia*	Mexico, Sinaloa, Altata	July 19, 1995	11	8,991 ± 7,946	0	Y	Y	n/a
C. circumpicta circumpicta	*Habroscelimorpha*	TX, Hardin Co., Sour Lake, Rt 105 × Rt 326	June 3, 1994	17	7,861 ± 8,677	0	Y	Y	n/a
C. circumpicta circumpicta	*Habroscelimorpha*	TX, Cameron Co., Port Isabel, Boca Chica Island	Sept 4, 1994	16	6,376 ± 4,356	0	Y	Y	n/a

TABLE 1. CONTINUED

Taxon	Subgenus	Locality	Date	*N*	Benzaldehyde (ng/beetle, mean ± SD)	Carryover	Not zero	Normal	Pearson et al.
C. circumpicta johnsoni	*Habroscelimorpha*	NM, Chaves Co., Roswell, Bottomless Lake SP	Aug 6, 1994	16	1,676 ± 1,983	0	Y	Y	–
C. cuprascens	*Ellipsoptera*	AL, Sumter Co., Epes, Tombigbee-Tennessee Waterway	June 28, 1995	10	3,295 ± 5,604	0	N	Y	–
C. dorsalis media	*Habroscelimorpha*	FL, Duval Co., Atlantic Beach, Little Talbot Island SP	June 21, 1994	16	1,065 ± 897	0	Y	Y	n/a
C. dorsalis saulcyi	*Habroscelimorpha*	FL, Escambia Co., Gulf Breeze, Gulf Islands NS	June 13, 1994	16	725 ± 661	0	Y	Y	n/a
C. dorsalis venusta	*Habroscelimorpha*	TX, Jefferson Co., Port Arthur, Sea Rim SP	June 6, 1994	16	918 ± 1,060	0	Y	Y	n/a
C. denverensis	*Cicindela*	NE, Scottsbluff Co., Scottsbluff, Wildcat Hills SRA	May 11, 1995	11	2,667 ± 4,950	0	N	Y	n/a
C. formosa generosa	*Cicindela*	IN, Porter Co., Dune Acres, Indiana Dunes SP	Sept 30, 1994	4	2,901 ± 1,050	0	Y	Y	–
C. formosa formosa	*Cicindela*	NE, Sheridan Co., Antioch, Rt 2	May 12, 1995	15	8,318 ± 6,250	0	Y	N	0
C. fulgoris albilata	*Habroscelimorpha*	TX, Hudspeth Co., Salt Flat, Salt Lake	Aug 4, 1994	27	1,352 ± 1,575	0	Y	Y	n/a
C. fulgoris erronea	*Habroscelimorpha*	AZ, Cochise Co., Wilcox, Wilcox Playa	Aug 15, 1994	16	1,675 ± 1,122	0	Y	Y	n/a

C. fulgoris fulgoris	*Habroscelimorpha*	NM, Sandoval Co., San Ysidro, Rio Salado	Aug 9, 1994	15	331 ± 281	0	Y	Y	0
C. gabbi	*Habroscelimorpha*	Mexico, Sonora, Bahia Kino	Aug 23, 1995	10	136 ± 261	7	N	Y	n/a
C. haemorragica haemorragica	*Cicindelidia*	NM, Sandoval Co., San Ysidro, Rt 44	Aug 9, 1995	15	20,366 ± 22,058	0	Y	Y	+ +
C. hamata monti	*Ellipsoptera*	TX, Jefferson Co., Port Arthur, Sea Rim SP	June 4, 1994	17	8,421 ± 9,384	0	Y	Y	–
C. hamata monti	*Ellipsoptera*	TX, Cameron Co., Port Isabel, Boca Chica Island	Sept 4, 1994	16	6,925 ± 6,219	0	Y	Y	–
C. hirticollis hirticollis	*Cicindela*	MD, St. Mary's Co., Mechanicsville, Cremona Farm	Sept 12, 1994	16	5,831 ± 4,732	0	Y	Y	+
C. hirtilabris	*Ellipsoptera*	FL, Duval Co., Atlantic Beach, Little Talbot Island SP	June 17, 1995	16	1,232 ± 2,105	0	Y	Y	n/a
C. lemniscata lemniscata	*Cylindera*	AZ, Cochise Co., Wilcox, Wilcox Playa	Aug 15, 1994	5	4,063 ± 2,968	0	Y	Y	–
C. lepida	*Ellipsoptera*	MS, Jones Co., Eastabuchie, Leaf River	June 26, 1995	16	5,560 ± 5,640	0	Y	Y	n/a
C. macrocnema macrocnema	*Opilidia*	Mexico, Nayarit, Bahia Matanchen	July 16, 1995	11	497 ± 283	0	Y	Y	n/a
C. marginata	*Ellipsoptera*	MD, St. Mary's Co., Sandgates, Patuxent River	July 8, 1994	15	4,317 ± 2,640	0	Y	Y	n/a
C. marutha	*Ellipsoptera*	AZ, Cochise Co., Wilcox, Wilcox Playa	Aug 13, 1994	15	2,912 ± 2,987	0	Y	Y	–

TABLE 1. CONTINUED

Taxon	Subgenus	Locality	Date	*N*	Benzaldehyde (ng/beetle, mean ± SD)	Carryover	Not zero	Normal	Pearson et al.
C. nevadica nevadica	*Ellipsoptera*	CA, San Bernadino Co., Baker, Mohavi NP	May 6, 1995	5	1,746 ± 1,705	0	N	Y	−*
C. nevadica olmosa	*Ellipsoptera*	NM, Sandoval Co., San Ysidro, Rio Salado	Aug 9, 1994	17	4,269 ± 5,311	0	Y	N	−*
C. ocellata rectilatera	*Cicindelidia*	TX, Hardin Co., Village Mills, Big Thicket NP	June 5, 1994	5	35,549 ± 32,290	0	N	Y	++
C. ocellata rectilatera	*Cicindelidia*	TX, Howard Co., Stanton, Red Lake	Aug 2, 1994	16	58,906 ± 55,272	0	Y	Y	++
C. oregona maricopa	*Cicindela*	NV, Clark Co., Mesquite, Virgin River	May 9, 1995	13	9,509 ± 8,911	0	Y	Y	+*
C. pamphila	*Habroscelimorpha*	TX, Kenedy Co., Corpus Christi, Padre Island NS	June 8, 1994	16	2,124 ± 1,935	1	Y	Y	−
C. pamphila	*Habroscelimorpha*	TX, Cameron Co., Port Isabel, Boca Chica Island	Sept 4, 1994	15	5,627 ± 4,448	0	Y	N	−
C. politula politula	*Cicindelidia*	TX, Schleicher Co., Elderado, Rt 277	Sept 1, 1994	16	5,408 ± 5,866	0	Y	Y	n/a
C. punctulata punctulata	*Cicindelidia*	TX, Hardin Co., Village Mills, Rt 420 × Rt 69	May 30, 1994	16	1,628 ± 2,857	0	Y	Y	−

C. punctulata punctulata	*Cicindelidia*	MD, St. Mary's Co., Mechanicsville, Cremona Farm	July 7, 1994	16	1,112 ± 814	0	Y	Y	–
C. repanda repanda	*Cicindela*	MD, Calvert Co., Honey Cove Estates, Patuxent River	July 14, 1994	17	17,500 ± 20,618	0	Y	Y	+
C. rockefelleri	*Habroscelimorpha*	Mexico, Sonora, La Choya	July 24, 1995	15	836 ± 741	0	Y	Y	n/a
C. severa severa	*Habroscelimorpha*	TX, Kenedy Co., Corpus Christi, Padre Island NS	June 10, 1994	17	5,726 ± 7,812	0	Y	Y	+
C. severa severa	*Habroscelimorpha*	TX, Cameron Co., Port Isabel, Boca Chica Island	Sept 4, 1994	16	5,846 ± 4,163	0	Y	Y	+
C. sexguttata sexguttata	*Cicindela*	TX, Hardin Co., Village Mills, Big Thicket NP	May 31, 1994	15	4,669 ± 4,769	0	Y	Y	0
C. sexguttata sexguttata	*Cicindela*	MD, St. Mary's Co., Cremona Farm	June 29, 1994	16	6,167 ± 6,311	0	Y	Y	0
C. scutellaris lecontei	*Cicindela*	IN, Porter Co., Dune Acres, Indiana Dunes SP	June 23, 1995	17	4,591 ± 3,953	0	Y	Y	–
C. sinaloae sinaloae	*Microthylax*	Mexico, Sinaloa, Altata	July 19, 1995	16	3,562 ± 2,456	0	Y	Y	n/a
C. sperata sperata	*Ellipsoptera*	TX, Howard Co., Stanton, Red Lake	Aug 2, 1994	15	6,937 ± 6,558	0	Y	Y	n/a
C. splendida	*Cicindela*	NE, Scottsbluff Co., Scottsbluff, Wildcat Hills SRA	May 11, 1995	1	3,070	0	–	–	0
C. striga	*Habroscelimorpha*	FL, Dixie Co., Jena, Rt 361	July 18, 1995	14	996 ± 1,227	2	Y	Y	n/a
C. tenuisignata	*Cicindelidia*	AZ, Yuma Co., Welton, Gila River	Aug 18, 1994	16	2,865 ± 2,677	0	Y	Y	n/a

TABLE 1. CONTINUED

Taxon	Subgenus	Locality	Date	*N*	Benzaldehyde (ng/beetle, mean ± SD)	Carryover	Not zero	Normal	Pearson et al.
C. togata fascinans	*Eunota*	NM, Chaves Co., Roswell, Bottomless Lake SP	Aug 7, 1994	15	581 ± 973	1	Y	Y	n/a
C. togata togata	*Eunota*	TX, Hardin Co., Village Mills, Rt 420 × Rt 69	May 31, 1994	16	80 ± 64	6	Y	Y	0
C. togata togata	*Eunota*	TX, Cameron Co., Port Isabel, Boca Chica Island	Sept 4, 1994	16	236 ± 180	0	Y	Y	0
C. tranquebarica moapana	*Cicindela*	UT, Kane Co., Mt. Carmel Jct., East Fork of Virgin River	Aug 25, 1994	16	1,483 ± 1,301	0	Y	Y	+, −*
C. trifasciata ascendens	*Cicindelidia*	Mexico, Sinaloa, Altata	July 19, 1995	14	32,072 ± 30,135	0	Y	Y	n/a
C. wapleri	*Ellipsoptera*	FL, Santa Rosa Co., Milton, Blackwater River SP	June 14, 1994	15	3,008 ± 3,358	0	Y	Y	n/a
C. wickhami	*Brasiella*	Mexico, Sinaloa, Barra de la Piaxtla	July 18, 1995	4	1,334 ± 717	0	Y	Y	n/a
Megacephala carolina		AL, Sumter Co., Epes, Tombigbee-Tennessee Waterway	June 28, 1995	14	176,140 ± 147,978	0	Y	Y	+

[a] Data includes the locality, date, number of samples, rank of Pearson et al. (1988), estimated benzaldehyde secreted (ng/beetle), number of samples less than the average carryover, and results of two statistical tests. Values from Pearson et al. (1988) marked by an asterisk (*) identity those taxa that differ in subspecies between the two studies. To determine whether the population mean could be distinguished from zero, Student's *t* tests were performed on the untransformed data (mean different from zero: $N = P > 0.05$, $Y = P < 0.05$). To assess whether the population fit a normal distribution, Shapiro-Wilk tests were performed on the log-transformed data (population normal: $Y = P > 0.05$, $N = P < 0.05$).

To ensure accurate quantification of benzaldehyde in beetle solutions, at least three dilutions of benzaldehyde in methylene chloride were included in each run (i.e., each set of samples placed in the 100 vial autosampler and analyzed). Using MS Chemstation software (HP G1034C), the samples of known concentration were used to establish a calibration curve for each run. The calibration curve was fitted using a quadratic regression forced through the origin because this method produced the minimum difference between the actual and estimated quantities of samples with known benzaldehyde concentration (Miller and Miller, 1993). All samples within a run were compared to this calibration curve for quantification, with the amounts of each detected ion summed to yield the quantity of benzaldehyde (nanograms per microliter) in the solution. The amount of benzaldehyde secreted per beetle (nanograms per beetle) was calculated by multiplying the measured quantity of benzaldehyde (nanograms per microliter) in each sample by a conversion factor (1000 μl/ml) and the solution volume (15 ml/beetle).

Statistics. A subset of samples was repeatedly assayed to assess repeatability of benzaldehyde measurement. Within- and between-run differences in the estimated amount of benzaldehyde were tested with univariate repeated measures analyses of variance. Intraclass correlations, using Pearson's product-moment correlation coefficient (Sokal and Rohlf 1981), also were calculated to evaluate the relationship between estimates of repeatedly measured samples. For all samples repeatedly assayed, only the first estimate of benzaldehyde was used in subsequent analyses.

Data from this study were compared with those of Pearson et al. (1988) to evaluate whether the studies differed in their estimates of benzaldehyde secretion for selected cicindelids. The mean nanogram per beetle values from this study and the relative estimates (0, $-$, $+$, and $++$) from Pearson et al. (1988) were used to rank species examined in both studies. Then these rankings were compared with a Wilcoxon's rank test.

Student's t tests were performed for each taxon sampled at a given locality to determine whether the population mean could be distinguished from zero (Sokal and Rohlf, 1981). For all subsequent analyses, amounts of benzaldehyde were natural log transformed to eliminate the association between the mean and standard deviation of taxon benzaldehyde estimates. Shapiro-Wilk tests of normality (Shapiro and Wilk, 1965) were performed to assess the benzaldehyde distributions of taxa. Taxonomic differences in benzaldehyde secretion were tested with a nested analysis of variance with the following nested groups: subgenus ($N = 9$), species ($N = 42$), subspecies ($N = 8$ species with >1 subspecies), and locality ($N = 8$ subspecies with >1 locality). Multiple comparisons of taxon means were performed using the Tukey-Kramer method (Tukey, 1953, cited in SAS Institute, Inc., 1989; Kramer, 1956) to control for

overall type I or experiment-wise error rate of comparisons. All analyses were conducted using PCSAS (SAS Institute, Inc., 1989).

RESULTS

Secretion from the pygidial gland occurred when the beetle was disturbed. Either a dark fluid oozed from the posterior end of the abdomen or an acrid-sweet smell was noted in the beetle's vicinity. The secretion was not forcibly ejected like that of bombardier beetles, but adhered to the tip of the abdomen. Secretion from the gland was accompanied by the beetle's repeatedly bending its abdomen tip. Both long pursuit and extensive handling of a beetle caused it to secrete benzaldehyde. On one very hot day the smell of benzaldehyde was noted near a large aggregation of *C. repanda*, although no samples were collected. It was not clear what had provoked secretion of the compound.

Carryover of benzaldehyde among samples was minimal. Of the 51 rinses used to assay carryover of beetle solutions among samples, only three showed any benzaldehyde (for all rinses: $\bar{X} = 0.002$ ng/μl, SD = 0.01). No taxon had a mean amount of benzaldehyde less than the average carryover and only 17 samples (2% of total) were estimated to have less benzaldehyde than this value.

Quantification of benzaldehyde in individual solutions was repeatable. Beetle solutions assayed more than once showed no difference in the quantity of benzaldehyde estimated to be present, whether the solution was sampled twice in a single run [mean square (MS) = 0.0046, $F_{1,11} = 1.62$, $P = 0.23$] or twice in different runs ($MS = 0.0001$, $F_{1,52} = 0.04$, $P = 0.85$). The intraclass correlations for solutions assayed twice within ($r = 0.937$, $N = 53$, $P < 0.001$) or between runs ($r = 0.998$, $N = 12$, $P < 0.001$) also demonstrate high repeatability of benzaldehyde quantification.

The amount of benzaldehyde varied greatly among individuals. The distribution of log transformed benzaldehyde quantities for all individuals ($N = 851$) was approximately normal (Shapiro-Wilk $W = 0.982$, $P = 0.052$; Figure 1), with a three order of magnitude range in amount secreted among members of the genus (Table 1). This large range of variation was similar to that found in the analysis of Pearson et al. (1988). No difference was found in the rankings of benzaldehyde secreted by taxa assayed in both this and Pearson's study (Wilcoxon's signed-rank test, $T_s = 127$, $P = 0.81$). The distributions of log-transformed benzaldehyde secretion also were normal for most of the taxa sampled (Table 1).

The amount of benzaldehyde secreted by beetles varied significantly among taxonomic units. Subgenera, species, and subspecies all accounted for significant portions of the total variation in benzaldehyde measured among individuals, while populations of subspecies sampled at different localities did not vary sig-

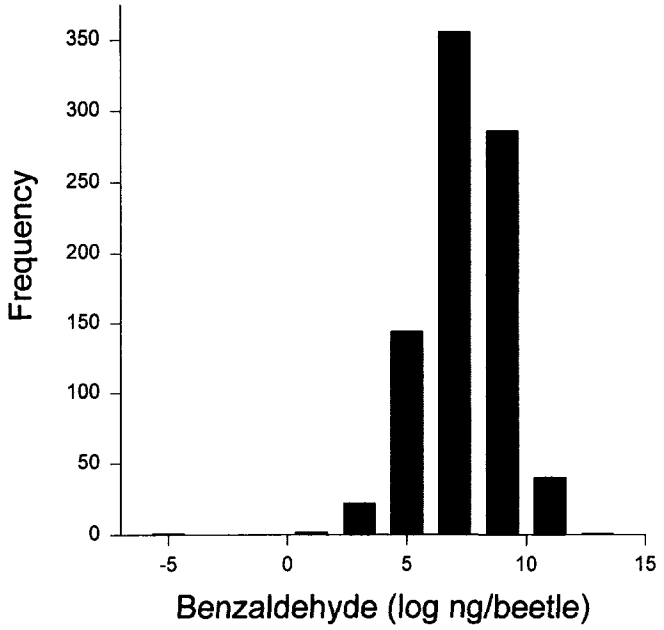

FIG. 1. Distribution of benzaldehyde among *Cicindela* samples (N = 851). Data were natural log transformed.

nificantly (Table 2). Members of the *Cicindela* subgenera *Cicindela*, *Cicindelidia*, *Microthylax*, and *Cylindera* secreted significantly more benzaldehyde than those of *Eunota*, while members of the remaining four subgenera (*Ellipsoptera*, *Habroscelimorpha*, *Brasiella*, and *Opilidia*) were intermediate in benzaldehyde

TABLE 2. NESTED ANALYSIS OF VARIANCE FOR AMOUNT OF BENZALDEHYDE SECRETED AMONG TAXONOMIC UNITS OF *Cicindela*[a]

Effect	*df*	*MS*	*F*	*P*	% Var
Subgenus	8	72.59	56.84	<0.001	20
Species	33	19.76	15.48	<0.001	21
Subspecies	10	7.53	5.89	<0.001	12
Locality	12	2.02	1.59	0.0904	2
Error	787	1.28			45

[a]Mean squares (*MS*) are based on type III sums of squares. Estimates of the variance components are expressed as a percentage of the total variance.

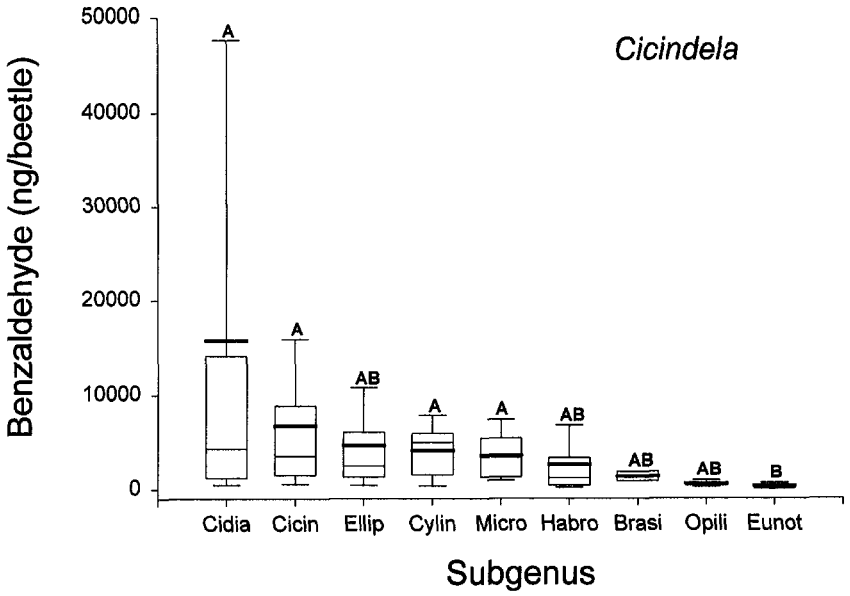

FIG. 2. Variation in benzaldehyde secretion among subgenera of *Cicindela*. Subgenera are ordered by mean benzaldehyde secreted and identified by the first five letters of their name, except *Cicindelidia* (=Cidia). Data presented are the mean (heavy line), median (thin line), 75% confidence limits (box), and 95% confidence limits (bars). Subgenera designated by different letters were significantly different in a multiple comparison test ($P < 0.05$).

secretion and could not be distinguished from these two groups (Figure 2). Interspecific comparisons within the *Cicindela* subgenera *Cicindela* and *Ellipsoptera* were more homogeneous (only two significantly different groups) in benzaldehyde secretion than were such comparisons in the *Cicindela* subgenera *Cicindelidia* and *Habroscelimorpha* (five significantly different groups; Figure 3). Finally, significant differences in benzaldehyde secretion among subspecies of *C.* (*Habroscelimorpha*) indicated that subspecific differentiation within some species has been accompanied by changes in the chemical defense of individuals (Figure 4).

DISCUSSION

Tiger beetles have long been known to produce fragrant or musky odors (Leng, 1902), well before benzaldehyde was identified in their pygidial glands.

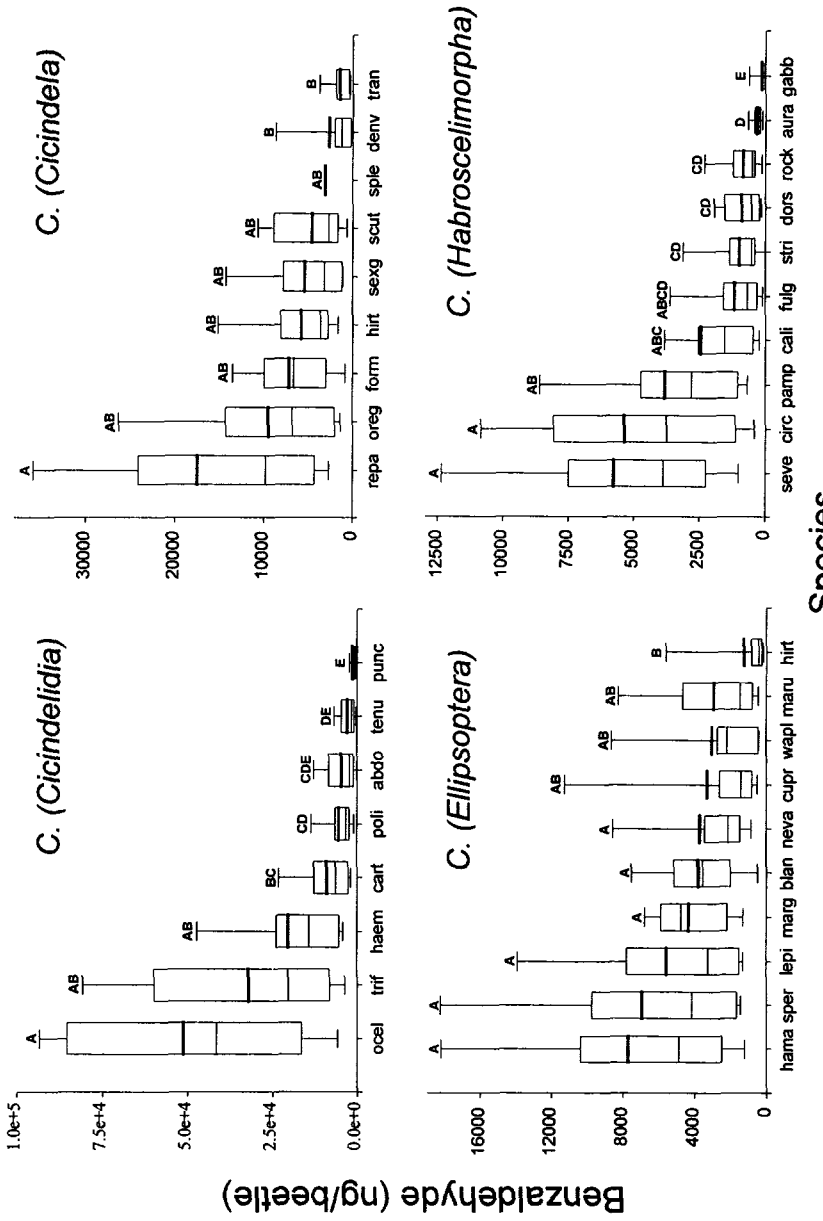

FIG. 3. Variation in benzaldehyde secretion among species of four subgenera. Species are identified by the first four letters of their names. Note the scale of benzaldehyde (nanograms per beetle) varies among subgenera to best illustrate the within-subgenus variation. Species designated by different letters were significantly different in a multiple comparison test ($P < 0.05$) within subgenera among species (pooled subspecies). All other conventions as in Figure 2.

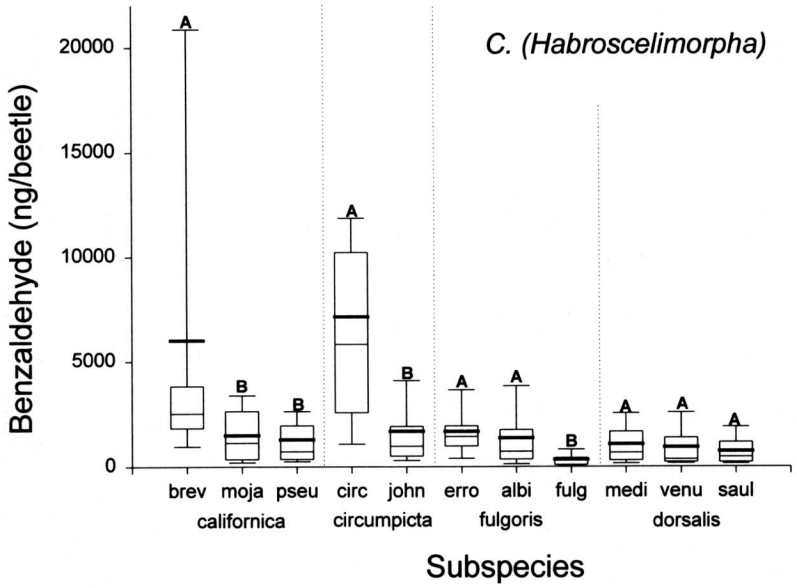

FIG. 4. Variation in benzaldehyde secretion among selected subspecies of *C. (Habroscelimorpha)*. Species are separated by dotted lines. Subspecies are identified by the first four letters of their names. Subspecies designated by different letters were significantly different in a multiple comparison test ($P < 0.05$) within species among subspecies (pooled populations). All other conventions as in Figure 2.

When the aromatic compound was first detected in the glandular secretions of individuals in the genus *Megacephala* (Moore and Brown, 1971; Moore, 1980; Blum et al., 1981), Moore and Brown (1971) doubted that members of the related genus *Cicindela* would produce benzaldehyde because of their relatively small pygidial reservoirs. In contrast, detailed anatomical studies have suggested ample development of the pygidial gland (Forsyth, 1970) and qualitative analyses of pygidial secretions from more than 83 species demonstrated that benzaldehyde is secreted by a taxonomically diverse array of *Cicindela* (Pearson et al., 1988).

In our study, benzaldehyde secretion was shown to vary over three orders of magnitude within the genus *Cicindela*. This range of variation was compatible with the absent, present, and abundant (0, +, and ++) assignments used by the previous comparative study (Pearson et al., 1988), as demonstrated by the similar ranking of species sampled in both studies. Our results also are comparable to those of Pearson et al. (1988) in that no consistent effect of locality

was found on benzaldehyde secretion, although a larger sample of populations might find such an effect significant. The estimated variance components (Table 2) indicate that the subgenus and species designations of a taxon have a considerably larger effect on benzaldehyde secretion than its subspecies or locality.

There are two important differences between this study and that of Pearson et al. (1988). Firstly, all species assayed in this study and previously designated as lacking benzaldehyde (0) or as secreting "trace or ambiguous" (−) amounts of benzaldehyde (Pearson et al., 1988) were shown to secrete small, but detectable, amounts of benzaldehyde. This suggests that all those species designated as lacking benzaldehyde by Pearson et al. (1988), in fact, do produce the compound; additional analyses are required to verify this suggestion. Secondly, there were some notable differences in the results obtained for individual taxa, particularly those for *C. hamata*, *C. formosa*, *C. sexguttata*, and *C. abdominalis*. The first three species were designated as having trace (−) or no benzaldehyde (0) by Pearson et al. (1988), whereas we found *C. hamata* and *C. formosa* secreted more benzaldehyde and *C. sexguttata* secreted only slightly less than two species designated as having benzaldehyde present (+), *C. hirticollis* and *C. severa*. Thus, Pearson et al. (1988) appear to have underestimated the amount of benzaldehyde secreted by these species. In the case of *C. abdominalis*, the previous study may have overestimated its amount of benzaldehyde (designated +), because we found this species secreted less than *C. hamata*, *C. formosa*, and *C. sexguttata*, three species designated as 0 or −. These discrepancies are likely the result of different techniques used in the field to collect the secretions. While Pearson et al. (1988) had several collectors obtain the beetle secretions and had more than one beetle put in a single sample bottle, we had a single individual (K.C.K.) involved in collecting all the secretions and put only one beetle in each bottle. Our techniques were designed to standardize secretion collection and minimize any differences in sample handling that might affect the analyses, and therefore may have yielded more precise measurements of benzaldehyde secretion. Additionally, sampling taxa at different localities or times of the year may have contributed to the difference in results.

Variation in benzaldehyde secretion was significantly related to the taxon's classification rank (subgenus, species, subspecies; Table 2). This result is consistent with those obtained by Pearson et al. (1988) and Mooi et al. (1989), who concluded there was strong evidence for a taxonomic (i.e., historical) effect on benzaldehyde secretion within the Cicindelidae. The current analysis cannot resolve the debate over the relative contributions of historical and ecological effects on benzaldehyde production, however, because the data still must be analyzed within a phylogenetic context (Mooi et al., 1989; Altaba, 1991; Vogler and Kelley, 1996). The ANOVA results indicate that meaningful phylogenetic analyses of the evolutionary changes in benzaldehyde secretion could be conducted among representatives of different subgenera (which may or may not be

monophyletic lineages) and among species and subspecies within a subgenus. Finally, with samples obtained from representatives at four potential levels of differentiation (subgenus, species, subspecies, locality), we postulate two specific hypotheses for the evolution of benzaldehyde production.

The first hypothesis concerns the mechanism of change in benzaldehyde production, particularly with regard to its apparent loss in some lineages (Pearson et al., 1988; Mooi et al., 1989; Altaba, 1991). In this study, all taxa assayed showed evidence of benzaldehyde production, suggesting that the gene(s) that code(s) for the synthesis of the compound is(are) not turned off or lost, as previously suggested (Mooi et al., 1989; Altaba, 1991). It is more likely that changes in the regulation of transcription from one or more of the pathway's gene(s) result in the increase or decrease of benzaldehyde production. Thus, the problem of restoring benzaldehyde production after its "loss" (Altaba, 1991) can be accomplished by changes in gene regulation, rather than the acquisition of a novel gene. Additionally, the observation that all taxa assayed showed evidence of producing benzaldehyde is most consistent with the hypothesis that there has been a single origin of the gene(s) required for the production of benzaldehyde within the cicindelid lineage (Blum et al., 1981; Dettner, 1987), contrary to other suggestions (Mooi et al., 1989).

The second hypothesis addresses the mode of change in benzaldehyde production, i.e., how selection may result in an increase or decrease in the synthesis of the compound. The observation that subspecies of a single species differed significantly in benzaldehyde secretion, while localities did not (within the limits of a test on populations of nine species), suggests that the process of differentiation among populations that results in morphologically distinct subspecies also may result in chemically distinct taxonomic units. If selection plays a role in the differentiation of cicindelid subspecies, then the same agent or associated agents of selection may be acting on the characters of chemical defense and morphology. It has been suggested that benzaldehyde production is under selection by robber flies (Pearson, 1985; Pearson et al., 1988), because the compound deters their attack (Pearson, 1985). If tiger beetles invade a new habitat or if the habitat in which they occur changes, one might expect a change in the risk of predation due to robber flies and other predators. This change in selection could result in modifications of a suite of characters associated with predator avoidance and deterrence, including chemical defenses, coloration, and body size or shape (Pearson, 1990; Kelley and Vogler, in preparation).

Previous studies have suggested that defensive secretions not derived exogenously should be quantitatively constant (Blum, 1981), but we found that variation within populations increased with the mean amount of benzaldehyde secreted ($r = 0.992$, $N = 41$, $P < 0.001$). There are two kinds of explanations for the large standard errors on the estimates of benzaldehyde secretion: experimental error and natural variation. First, experimental error may have increased

the variance among samples if some beetles secreted significant amounts of benzaldehyde before they were put in the bottle of solvent or if there was significant evaporation of the solution between the time of collection and analysis. These errors were minimized by setting a limit on the time to putting the beetle in the bottle and by keeping the solutions cool; however, beetles naturally vary in the amount of time and handling it takes for them to release their pygidial secretions, and evaporation could not be accounted for without an internal standard. While these sources of error should not yield the observed positive association between the mean and variance in compound secretion, it is not unusual to find such a positive association in measured biological variables (Sokal and Rohlf, 1981). Thus, the observed variance more likely reflects real variance in the amount of compound an individual had to secrete at any given time (Pasteels et al., 1983). This variance may result from a time lag in regenerating the compound after a beetle has secreted previously (Hill and Tschinkel, 1985) or from genetic variation in the amount of the compound individuals can produce (Engelhardt et al., 1965). Additionally, variance in the secretion of chemical defenses can depend on an individual's sex (Blum, 1981; Hill and Tschinkel, 1985; Whitman et al., 1992), age (Blum, 1981; Hill and Tschinkel, 1985; Pearson et al., 1988; Whitman et al., 1992), body mass (Whitman et al., 1992), or resource availability (Pearson et al., 1988; Whitman et al., 1992). The relative contributions of these factors to variation in benzaldehyde secretion could be compared with taxonomic effects on chemical defense in repeated assays of live captive beetles.

The quantification of benzaldehyde secreted by individual beetles makes it possible to identify an appropriate range of compound concentrations to be used in predation experiments. The amount of benzaldehyde that a beetle secretes in response to a potential predator is likely to be equivalent to or less than that measured, because the methylene chloride extraction is thought to cause the release of the pygidial gland's entire contents (Pearson et al., 1988). This assumption could be tested by assessing the relationship between the amount of benzaldehyde measured with destructive (used here and in Pearson et al., 1988) and live-beetle assays. Assuming that the destructive sampling technique provides an adequate estimate of benzaldehyde secretion in cicindelids, the average amount of benzaldehyde secreted by an individual *C. ocellata rectilatera* (51 μg), the taxon with the highest benzaldehyde measured, was almost nine times more than the amount sufficient to repel predatory ants (6.4 μg) (Peterson, 1986), but was three orders of magnitude less than the amount reported to repel robber flies and lizards (average amount = 36 mg) (Pearson, 1985). This difference between the observed amount of benzaldehyde secreted by tiger beetles and that used in experimental presentations may limit the conclusions of Pearson (1985) to the statement that extremely large amounts of benzaldehyde deter robber flies. It is not known whether lower, more ecologically relevant concen-

trations of benzaldehyde are equally effective at deterring robber flies. However, as Whitman (1988) has pointed out, insects encounter a variety of predators which vary in their ability to handle prey defensive compounds; thus, even a slight increase in chemical defense should deter some additional predators. Indeed, the amount of benzaldehyde secreted by some tiger beetle species may be enough to repel some birds (Clark and Shah, 1994). Future studies evaluating the deterrent effect of benzaldehyde on potential predators should use concentrations of the compound that span the observed range of benzaldehyde secreted by tiger beetles.

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SOLID-PHASE MICROEXTRACTION AND CUTICULAR HYDROCARBON DIFFERENCES RELATED TO REPRODUCTIVE ACTIVITY IN QUEENLESS ANT *Dinoponera quadriceps*

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Abstract—We extracted the cuticular hydrocarbons from live *Dinoponera quadriceps* ants (10 colonies collected from Brazil) with the solventless solid-phase microextraction (SPME) technique. Gas chromatography of the SPME samples (N = 233 measurements) compared with pentane extracts (N = 10) resulted in similar profiles. Eighty-one compounds belonging to the main long-chain hydrocarbon families were identified by GC-MS. There is no morphologically specialized queen in *D. quadriceps* and only one aggressively dominant worker (alpha) mates and reproduces in each colony. The alpha ant (N = 26 individuals) always yielded higher amounts and percentages of 9-hentriacontene (9-C_{31} : 1) than her sterile nestmates (N = 47). Since SPME is not destructive, it allowed for the repeated extraction of the same individuals, demonstrating that the alpha ant (virgin or mated) always had higher levels of 9-hentriacontene. This difference appears related to ovarian activity and may function as a signal of the alpha's dominance status.

Key Words—Ant, Ponerinae, reproduction, dominance, cuticular hydrocarbons, solid-phase microextraction.

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INTRODUCTION

All insect societies are distinguished by the sterility of most group members. In species where reproductive and sterile individuals are morphologically specialized (termites, most ants, some bees and wasps), queens produce pheromones regulating the egg-laying of workers and their rearing of new female sexuals (Fletcher and Ross, 1985). However, queen pheromones have only been identified in honeybees, where they are produced in the mandibular glands (Winston, 1987; Plettner et al., 1996). In contrast, in social insects lacking physical castes, reproduction is regulated by aggressive interactions among a proportion of nestmates (Heinze et al., 1994). This aggression is highly directed and necessitates the olfactory recognition of dominance status (West-Eberhard, 1977; Downing and Jeanne, 1985).

About 100 species belonging to 10 genera of the ant subfamily Ponerinae have lost the queen caste (Peeters, 1993). All workers are morphologically similar, but according to species either one or several mate and lay fertilized eggs in each colony. In the monogynous *Dinoponera quadriceps*, only the worker having top-rank in the hierarchy ("alpha") can mate (Monnin and Peeters, 1998). Almost all subordinates have inactive ovaries. The alpha ant exhibits an exceptional dominance behavior. She bends her gaster forward, bites the tip of one antenna of a subordinate worker and rubs it against the intersegmental membranes between her abdominal tergites V and VII. This behavior is likely to involve the transfer of olfactory information. Our aim was to identify the cuticular hydrocarbons and determine whether they differ between the fertile alpha and sterile subordinate workers.

Solid-phase microextraction (SPME) (Berlardi and Pawliszyn, 1989; Arthur and Pawliszyn, 1990, Arthur et al., 1992) seemed an ideal technique to study cuticular hydrocarbons of *D. quadriceps*. Since a limited number of colonies were available for study (with only one alpha in each), a nondestructive technique was needed. SPME was recently used to document the daily production of a volatile pheromone in the curculionid beetle *Metamasius hemipterus* (Malosse et al., 1995) and to extract directly a pheromone from an exocrine gland in Lepidoptera, thereby avoiding solvent interaction (Frérot et al., 1997, Mozuraitis et al., 1996).

METHODS AND MATERIALS

Colonies of *Dinoponera quadriceps* (82 $\pm$ 29 workers; range 39–141; N = 17) were collected in Bahia state (along the road between Sambaiba and Tobias Barreto), Brazil, in October 1994 and January 1996. They were kept in artificial laboratory nests where live insects were provided daily as food. Ten

colonies were used for chemical analyses; all the workers had been individually marked with numbers glued onto their thorax, and aggressive interactions between nestmates were recorded in order to determine their rank in the hierarchy. Several workers eclosed from their cocoons in the laboratory, and thus their exact age was known. All workers were later dissected to check their ovarian activity and presence of sperm in the spermatheca (Monnin and Peeters, 1998). Since the alpha ant is able to oviposit before she has mated, we differentiate between virgin alphas and mated alphas (the latter are termed gamergates) (Peeters, 1993).

Cuticular hydrocarbons of individual workers were sampled with a Supelco 7-μm polydimethylsiloxane fiber, designed to extract compounds of high molecular weight. Live ants were immobilized with a nylon wire and entomological pins. The sting was seized with forceps to gently bend the abdomen underneath the thorax, thus exposing the sclerotized membrane between tergites VI and VII; this is where dominant workers normally rub the antennae of subordinates. The fiber was carefully rubbed against this membrane for 2 min, then desorbed in the injection port of a gas chromatograph for 5 min, for either GC or GC-MS analysis. *Dinoponera* workers are the largest known ants (roughly 3 cm in length), which facilitated our sampling procedure. There were no injuries because intersegmental membranes are very thick in ponerine species; in fact, the flexible SPME fiber broke readily. Seventy-three workers of different ages, ranks, and colonies were measured with SPME-GC. Since SPME is not destructive, we were able to sample most workers several times (Table 1).

We attempted to estimate the absolute quantities of hydrocarbons extracted. Addition of an internal standard to the SPME samples was impossible: rubbing the fiber on the ant after adding the standard would have changed the amount

TABLE 1. NUMBER OF SPME PERFORMED ON WORKERS FROM 10 COLONIES[a]

Functional group	Individuals (*N*)	Total SPME samples	SPME samples per individual	
			Mean ± SE	Min–Max
Mated alphas	7	60	8.6 ± 2.1	1–17
Virgin alphas	19	85	4.5 ± 0.8	1–14
Young sterile workers (less than 1 month-old)	11	25	2.3 ± 0.4	1–5
Older sterile workers (more than 1 month-old)	36	63	1.8 ± 0.2	1–6

[a] Alphas (mated or virgin) had this rank for more than two weeks.

of standard finally injected, and immersing the fiber in the standard after sampling the ant would have changed the amount of extract injected. Thus, we injected 3 μg of n-C_{31} in the GC-MS as an external standard ($N = 2$), and the area of the n-C_{31} peak allowed us to estimate the quantity corresponding to the area of each peak in the chromatograms. SPME of the head, petiole, and abdomen of the same alpha workers was done to compare the spatial distribution of hydrocarbons over the body. This was replicated with 12 alphas (mated or virgin) from six colonies. The Dufour's gland of five alphas and nine subordinate workers was dissected. Each gland was extracted in 200 μl of pentane for at least 24 hr, and 5 μl of the solution was injected for GC analysis.

Our sampling protocol is a novel application of SPME and thus needed validation. Classical pentane extractions of three alphas and seven subordinates were done for comparison. Each ant was cooled at 5°C for a few minutes and then totally immersed in 2 ml of pentane for 2 min. Five microliters of the extract were injected for GC or GC-MS analysis. We also compared the efficiency of SPME and pentane in extracting a reference mixture previously applied on a dead ant. The mixture was composed of 9-C_{23}:1, n-C_{23}, n-C_{26}, n-C_{28}, n-C_{30}, n-C_{32}, n-C_{34}, and n-C_{36} at 63, 160, 26, 21, 57, 77, 27 and 19 ng/μl, respectively. These standard compounds are present in low amounts on the ants' cuticle, thus avoiding interference with the compounds interest. The dead ant was washed with pentane, after which 10 μl of the reference mixture were applied on her abdomen. After a few seconds needed for solvent evaporation, the abdomen was sampled with the SPME fiber for 1 min and analyzed. This procedure was then repeated ($N = 11$), except that after some SPME measurements, the ant was washed with 500 μl of pentane for 2 min. This extract was evaporated to 50 μl, and 2 μl was injected for chemical analysis ($N = 4$).

GC analyses were conducted with a HP 5890 Series II chromatograph equipped with a split–splitless injector and a FID detector heated at 260°C. The nonpolar fused-silica capillary column (HP-5, Hewlett Packard, 30 m × 0.32 mm ID, 0.25-μm film phase) was programmed from 260°C (isothermal for 15 min) to 300°C at 5°C/min, then isothermal for 22 min, with helium as carrier gas at 15 psi. The integrations were realized with HP GC-ChemStation Software. Combined gas chromatography–mass spectrometry (GC-MS) was done with either SPME samples or pentane samples, using a Varian 3300 gas chromatograph equipped with a SPI injector heated at 280°C, and linked to a Nermag R10-10C quadrupole mass analyzer piloted by HP GC1034C ChemStation Software. Compounds were eluted on a 25-m × 0.32-mm-ID, 0.4-μm film-phase, nonpolar fused-silica capillary column (DB5-MS, J & W Scientific) programmed from 200 to 310°C at 5°C/min. Spectral data were obtained with electronic impact (EI, 40–550 amu). Chemical ionization (CI) was performed with methyl-vinyl-ether (MVE) (Matheson Gas Products) scanning from 130 to 550 amu.

To estimate the similarity of the hydrocarbon profiles obtained from different SPME-GC measurements of the same individuals, we computed the coefficients of variation (standard deviation divided by mean) for each of the major peaks. We selected six mated alphas that had been measured five times each (over a few weeks), and compared coefficients of variation within and between individuals.

The hydrocarbon profiles obtained by SPME-GC were compared among four functional groups: mated alphas, virgin alphas, young sterile workers, and older sterile workers (which includes foragers). Fourteen major peaks occurring regularly were selected for statistical analysis. The percentages of each peak were compared between groups by an analysis of variance (ANOVA). Since most workers had been sampled several times, we used the mean percentage of each peak. Differences between groups were further investigated with a discriminant analysis. Since relative proportions are dependent variables, they cannot be used for discriminant analysis. Thus, to avoid the limitations inherent to the analysis of compositional data, peak areas were standardized according to the formula of Reyment (1989):

$$Z_{i,j} = \ln [Y_{i,j}/g(Y_j)]$$

where $Y_{i,j}$ is the area of peak i for ant j, $g(Y_j)$ the geometric mean of the areas of all peaks for ant j, and $Z_{i,j}$ the standardized area of peak i for ant j. For workers measured several times, we used the mean standardized areas of each peak. Profiles where one of the peaks had a null area were removed, and thus the discriminant analysis was performed on 57 workers instead of 73 used for the ANOVA. Factor structure coefficients, which are the correlations between each peak and the factors of the discriminant analysis, were plotted to interpret which peak contributes most to the separation between the four groups of workers.

RESULTS

A GC-MS analysis of a pentane extract of the cuticle from an alpha worker is shown in Figure 1, where compounds are numbered in order of elution on the nonpolar column. Eighty-one compounds belonging to the main long-chain hydrocarbon families and ranging from 21 to 37 carbon atoms were identified (Table 2): saturated, mono- and diunsaturated, and methyl-branched alkanes. All methyl-branched alkanes were determined by comparison of equivalent chain lengths (ECL) and diagnostic ion fragments (Table 2) with that of alkanes in other ant species (Lenoir et al., 1997).

Hydrocarbon profiles obtained by SPME-GC are similar, except that there are fewer peaks (Figure 2). Alphas and subordinate workers differ remarkably

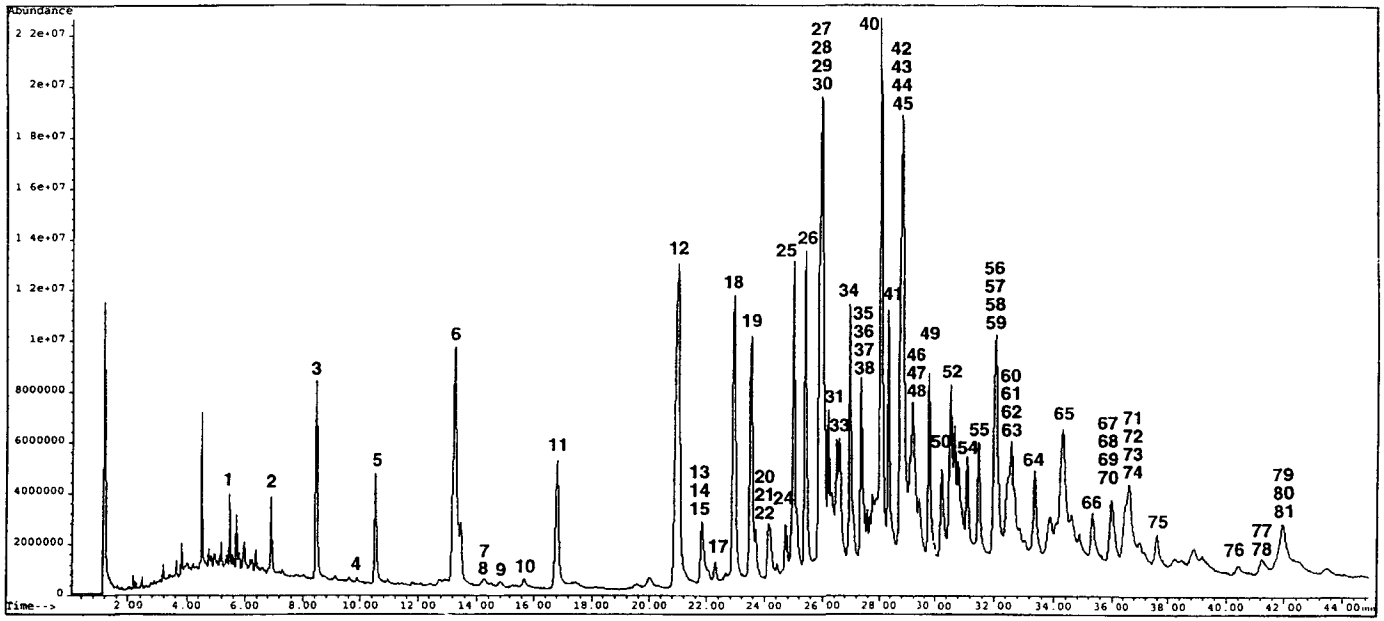

FIG. 1. GC-MS chromatogram of a virgin alpha extracted in pentane. Compounds are identified in Table 2.

TABLE 2. COMPOUNDS IDENTIFIED IN CUTICULAR PENTANE EXTRACT OF VIRGIN ALPHA WORKER, TOGETHER WITH PHYSICOCHEMICAL DATA[a]

No.	Component	ECL	Mol wt	Diagnostic EI ions
1	*n*-C_{21}	21.00	296	296
2	*n*-C_{22}	22.00	310	310
3	*n*-C_{23}	23.00	324	324
4	3-MeC_{23}	23.69	338	56, 308/309
5	*n*-C_{24}	24.00	338	338
6	*n*-C_{25}	25.00	352	352
7	11-MeC_{25}	25.28	366	168/169, 224/225
8	13-MeC_{25}	25.28	366	196/197
9	5-MeC_{25}	25.44	366	84, 308/309
10	3-MeC_{25}	25.69	366	56, 336/337
11	*n*-C_{26}	26.00	366	366
12	*n*-C_{27}	27.00	380	380
13	9-MeC_{27}	27.32	394	140/141, 280/281
14	11-MeC_{27}	27.32	394	168/169, 252/253
15	13-MeC_{27}	27.32	394	196/197, 224/225
16	7-MeC_{27}	27.41	394	112, 308/309
17	5-MeC_{27}	27.51	394	84, 336/337
18	3-MeC_{27}	27.77	394	56, 364/365
19	*n*-C_{28}	28.00	394	394
20	9-MeC_{28}	28.33	408	140/141, 294/295
21	11-MeC_{28}	28.33	408	168/169, 266/267
22	13-MeC_{28}	28.33	408	196/197, 238/239
23	5-MeC_{28}	28.48	408	84, 350/351
24	3-MeC_{28}	28.78	408	56, 378/379
25	9-C_{29}:1	28.79	406	138/170*, 292/324*
26	*n*-C_{29}	29.00	408	408
27	9-MeC_{29}	29.35	422	140/141, 308/309
28	11-MeC_{29}	29.35	422	168/169, 280/281
29	13-MeC_{29}	29.35	422	196/197, 252/253
30	7-MeC_{29}	29.41	422	112, 336/337
31	5-MeC_{29}	29.51	422	84, 364/365
32	11,15-diMeC_{29}	29.58	436	168, 295, 239, 224
33	13,17-diMeC_{29}	29.58	436	196, 267
34	3-MeC_{29}	29.73	422	56, 392/393
35	*n*-C_{30}	30.00	422	422
36	11-MeC_{30}	30.31	436	168/169, 294/295
37	12-MeC_{30}	30.31	436	182/183, 280/281
38	13-MeC_{30}	30.31	436	196/197, 266/267
39	14-MeC_{30}	30.31	436	210/211, 252/253
40	9-C_{31}:1	30.80	434	138/170*, 320/352*
41	*n*-C_{31}	31.00	436	436
42	9-MeC_{31}	31.32	450	140/141, 336/337
43	11-MeC_{31}	31.32	450	168/169, 308/309
44	13-MeC_{31}	31.32	450	196/197, 280/281

TABLE 2. CONTINUED

No.	Component	ECL	Mol wt	Diagnostic EI ions
45	15-MeC_{31}	31.32	450	224/225, 252/253
46	9,13-diMeC_{31}	31.57	464	140, 351, 211, 280
47	11,15-diMeC_{31}	31.57	464	168, 323, 239, 252
48	13,17-diMeC_{31}	31.57	464	196, 295, 267, 224
49	*n*-C_{32}	32.00	450	450
50	12-MeC_{32}	32.27	464	182/183, 308/309
51	14-MeC_{32}	32.27	464	210/211, 280/281
52	2Δ-C_{33}:2	32.44	460	460
53	2Δ-C_{33}:2	32.52	460	460
54	9-C_{33}:1	32.77	462	138/170*, 348/380*
55	*n*-C_{33}	33.00	464	464
56	11-MeC_{33}	33.32	478	168/169, 336/337
57	13-MeC_{33}	33.32	478	196/197, 308/309
58	15-MeC_{33}	33.32	478	224/225, 280/281
59	17-MeC_{33}	33.32	478	252/253
60	9,11-diMeC_{33}	32.58	492	140, 379, 211, 308
61	11,15-diMeC_{33}	32.58	492	168, 351, 239, 280
62	13,17-diMeC_{33}	32.58	492	196, 323, 267, 252
63	15,19-diMeC_{33}	32.58	492	224, 295
64	*n*-C_{34}	34.00	478	478
65	2Δ-C_{35}:2	34.48	488	488
66	*n*-C_{35}	35.00	492	492
67	11-MeC_{35}	35.29	506	168/169,364/365
68	13-MeC_{35}	35.29	506	196/197, 336/337
69	15-MeC_{35}	35.29	506	224/225, 308/309
70	17-MeC_{35}	35.29	506	252/253, 280/281
71	9,13-diMeC_{35}	35.57	520	140, 407, 211, 336
72	11,15-diMeC_{35}	35.57	520	168, 379, 239, 308
73	13,17-diMeC_{35}	35.57	520	196, 351, 267, 280
74	15,19-diMeC_{35}	35.57	520	224, 323, 295, 252
75	*n*-C_{36}	36.00	506	506
76	*n*-C_{37}	37.00	520	520
77	13-MeC_{37}	37.31	534	196/197, 364/365
78	15-MeC_{37}	37.31	534	224/225, 336/337
79	11,15-diMeC_{37}	37.58	548	168, 407, 239, 336
80	13,17-diMeC_{37}	37.58	548	196, 379, 267, 308
81	15,19-diMeC_{37}	37.58	548	224, 351, 295, 280

[a] Diagnostic ions marked with an asterisk were used to locate the double bond position.

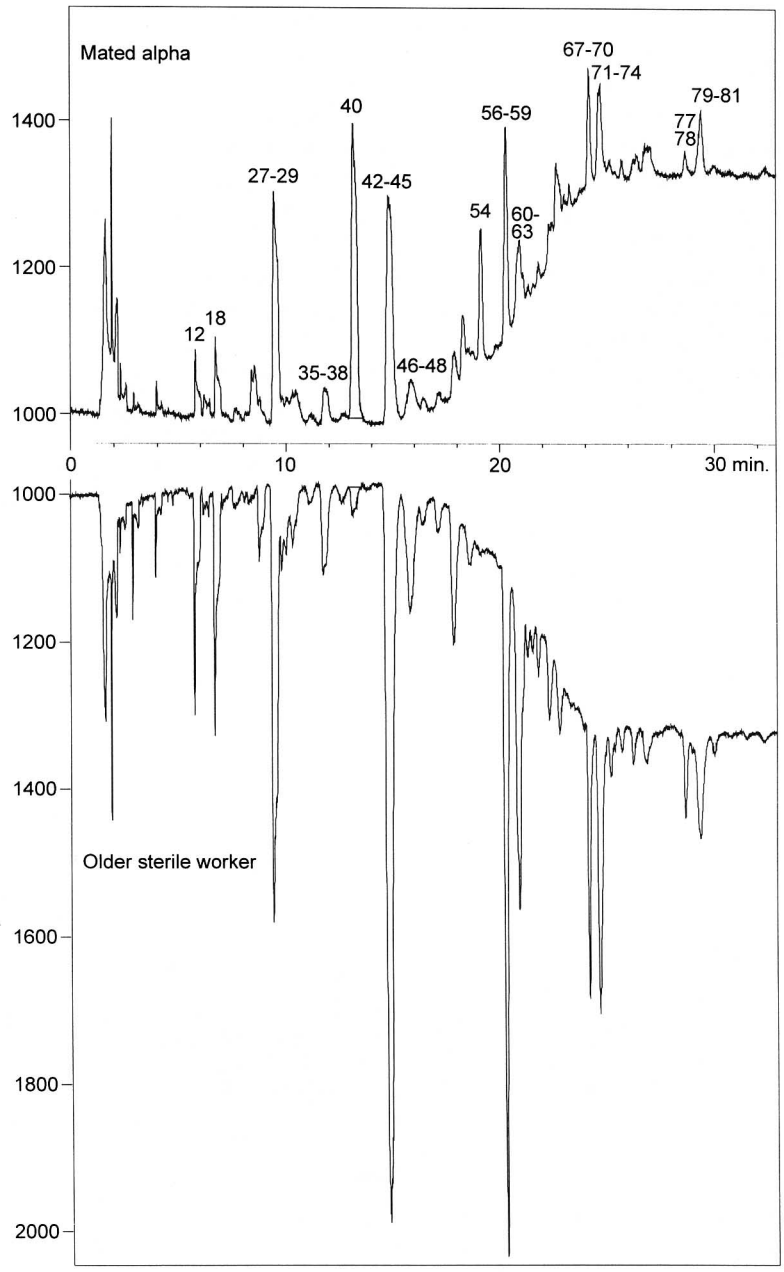

FIG. 2. SPME-GC chromatograms of a mated alpha (above) and an older sterile worker (below). Note the difference for peak 40 (9-hentriacontene), characteristic of alpha workers. Quantitative details are given in Table 3.

by the amount of peak 40. EI mass spectrum of this peak is characteristic of a monounsaturated alkane with 31 carbon atoms [ions at *m/z* 55, 69, 83, 97, 111, 434 (M^+)]. The double bond position was determined directly from the ion-molecule reaction with MVE (Ferrer-Correia et al., 1976; Malosse et al., 1994). In the GC-(MVE)-CI/MS mass spectrum, in addition to molecular ion species [*m/z* 432 $(M-2H)^+$ and 493 $(M+HMVE)^+$], diagnostic ions at *m/z* 138/170 and 320/352 locate unambiguously the double bond at a C_9 position (9-hentriacontene, or 9-C_{31}). These two pairs of ions, both separated by 32 amu, are related to the position of the double bond in the initial alkene. The C_{29} and C_{33} alkenes present in the extracts were identified in the same way (diagnostic ions marked with an asterisk in Table 2).

SPME is nondestructive and thus allowed for multiple extractions of the same individuals (Table 1). To examine the repeatability of SPME measurements, we compared coefficients of variation within and between individuals (shown for six mated alphas in Figure 3). Intraindividual variation was lower than interindividual variation for most major peaks, except those in the higher range of the chromatographic profiles. This indicates that our measurements are reliable to group individuals according to the similarity of their profiles.

After assigning individual workers to four arbitrary groups (based on age

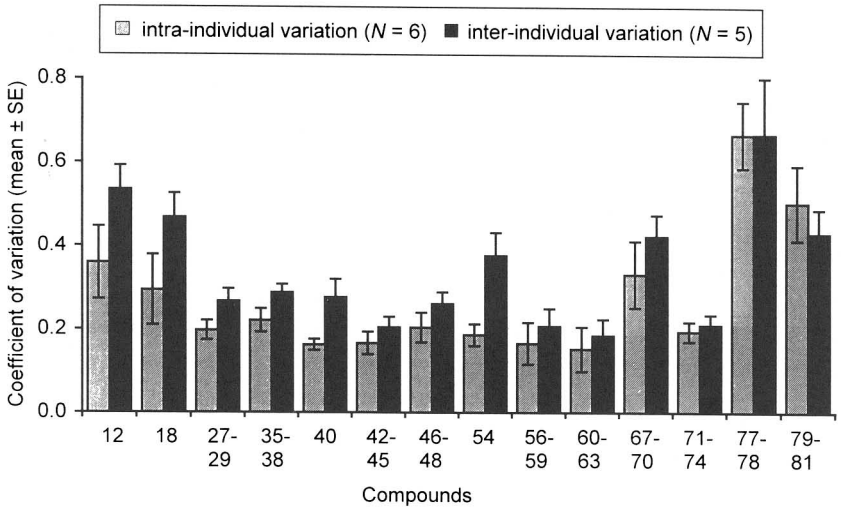

FIG. 3. Repeatability of SPME measurements for each of the 14 major peaks. A coefficient of intraindividual variation was computed with five repeated measurements for each of six alphas. A coefficient of interindividual variation was computed using one of the measurements for each alphas, and this was repeated five times.

and dominance characteristics), we used a discriminant analysis to compare their cuticular hydrocarbon profiles (based on the areas of 14 major peaks). Our biological groupings were retained (Figure 4), revealing a link with cuticular differences. Three groups were clearly separated: alphas (both virgin and mated), young sterile workers, and older sterile workers. The factor structure coefficients indicated that 9-C_{31} contributes most to the discrimination between these groups along factor 1 (74% of the variance). The ANOVA confirmed that alphas yield higher percentages of 9-C_{31} than subordinate workers (Table 3). Although the discriminant analysis did not separate mated alphas from virgin alphas (Figure 4), the ANOVA found that mated alphas have higher percentages of 9-C_{31}. Peak 54 (9-tritriacontene) discriminated both alphas and young sterile workers from older workers along factor 1, and young sterile workers from alphas along factor 2 (20% of the variance) (Figure 4). Further evidence that this compound is not characteristic of alphas is its higher relative proportion in young sterile workers (Table 3). When the discriminant analysis was repeated without 9-C_{31} and 9-tritriacontene, the worker groups were no longer separated (Figure 5), indicating that no other compounds can discriminate alphas from sterile workers.

SPME of alpha workers revealed that the proportion of 9-C_{31} does not differ between head, petiole, and abdomen (6.6 $\pm$ 0.5, 7.1 $\pm$ 0.6, and 7.8 $\pm$ 0.7%, respectively; $N = 12$, ANOVA: $P = 0.205$). However, all compounds were present in higher quantities on the abdomen than on the head and the petiole (704 $\pm$ 88, 367 $\pm$ 60, and 380 $\pm$ 39 ng, respectively; ANOVA, post-hoc comparison: Scheffé test: $P < 0.01$). 9-C_{31} was not found in the Dufour's gland of five alpha workers (identified products included saturated, mono- and diunsaturated hydrocarbons from n-C_{14} to n-C_{23}), whereas in two of these workers, pentane washes of the cuticle yielded 9.3 and 9.8% of 9-C_{31}.

Comparison of SPME ($N = 11$) and pentane extractions ($N = 4$) of the reference mixture indicated that pentane gives the closest profiles, while SPME preferentially extracted some shorter-chain hydrocarbons (43% vs 26% for n-C_{23} with SPME and pentane, respectively) and underextracted longer-chain hydrocarbons (11, 13, 4, and 3% vs. 16, 24, 8, and 5% for n-C_{30}, n-C_{32}, n-C_{34}, and n-C_{36} with SPME and pentane, respectively). The difference was significant at $P < 0.05$ for n-C_{36} and $P < 0.01$ for the other compounds (Mann-Whitney U test). In contrast, SPME ($N = 26$) and pentane extraction ($N = 3$) of alpha workers revealed that SPME underestimated shorter-chain compounds (No. 12, 18, 27–29, 40) and overestimated longer-chain compounds (No. 54, 56–59, 60–63, 67–70, and 71–74). Extraction of older sterile workers by SPME ($N = 36$) and pentane ($N = 7$) showed similar tendencies, but the percentages of each peak did not differ significantly (Mann-Whitney U test: $P > 0.05$). Both SPME and pentane extracted approximately seven times more 9-C_{31} on alphas than on sterile workers. SPME measured 53 $\pm$ 6 vs. 7 $\pm$ 1 ng for alphas

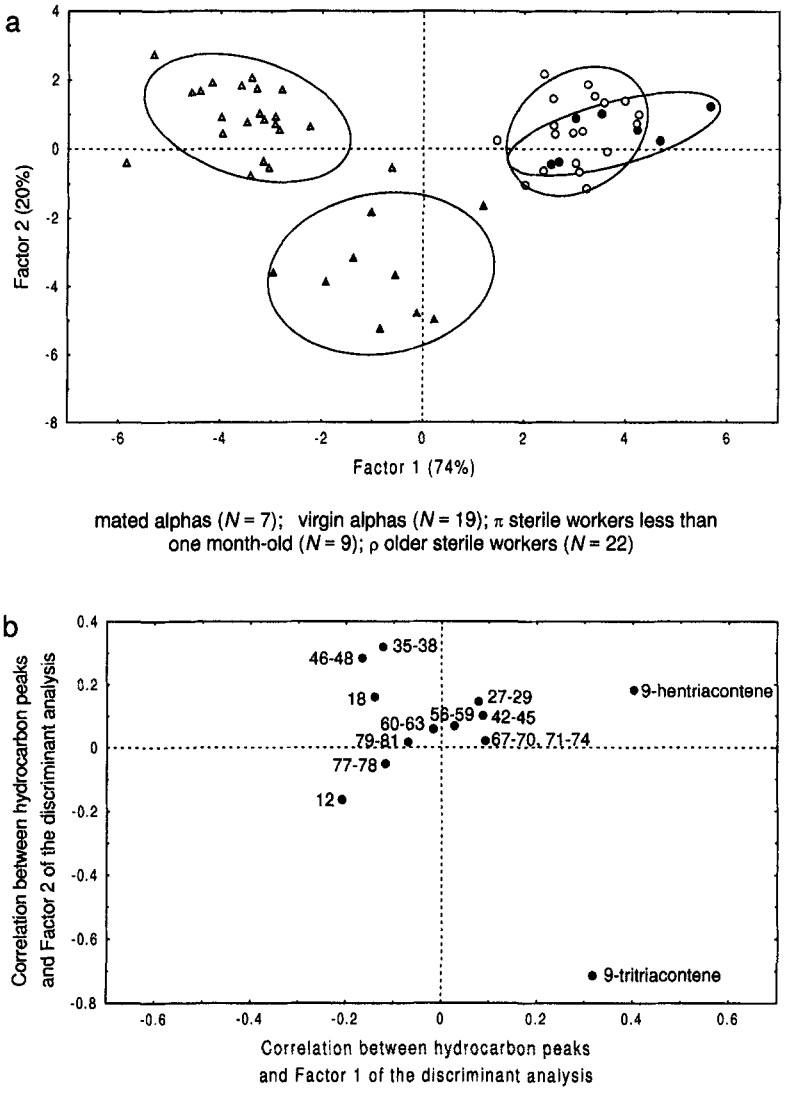

FIG. 4. Discriminant analysis of 57 workers assigned to four functional groups. The areas of 14 major peaks were compared (after standardization). Each data point represents the mean measure for one individual in one group (two workers switched groups). Envelopes represent the 95% confidence ellipses. Factor structure coefficients are indicated in lower graph.

TABLE 3. MEAN PERCENTAGES OF 14 MAJOR PEAKS (±SE) OBTAINED BY 233 SPME-GC OF 73 WORKERS[a]

Compound number	Mated alphas (N = 7)	Virgin alphas (N = 19)	Young sterile workers (N = 11)	Older sterile workers (N = 36)
12	2.9 ± 0.4	3.1 ± 0.2	3.5 ± 0.7	3.6 ± 0.3
18	3.4 ± 0.5	4.4 ± 0.3	3.3 ± 0.6	4.2 ± 0.3
27-29	10.0 ± 0.8ab	11.7 ± 0.7a	7.0 ± 1.0b	10.5 ± 0.7ab
35-38	2.0 ± 0.1ab	2.3 ± 0.1a	1.5 ± 0.2b	2.4 ± 0.1a
40	9.7 ± 0.9a	7.3 ± 0.7b	1.7 ± 0.3c	0.8 ± 0.1c
42-45	13.9 ± 0.7ab	15.2 ± 0.7ab	11.0 ± 1.2b	17.2 ± 0.8a
46-48	3.0 ± 0.2ab	3.1 ± 0.2a	1.9 ± 0.3b	3.0 ± 0.2ab
54	3.1 ± 0.3ac	2.3 ± 0.2a	5.0 ± 0.9c	0.4 ± 0.1b
56-59	7.9 ± 0.2	7.5 ± 0.2	6.9 ± 0.7	8.3 ± 0.4
60-63	6.3 ± 0.4	6.3 ± 0.2	6.2 ± 0.6	7.4 ± 0.2
67-70	19.0 ± 2.3	18.4 ± 2.0	28.4 ± 3.8	23.0 ± 2.1
71-74	13.0 ± 1.2ab	12.4 ± 0.7a	16.1 ± 1.1b	12.2 ± 0.4a
77-78	1.8 ± 0.2	1.8 ± 0.2	2.6 ± 1.1	2.8 ± 0.5
79-81	4.0 ± 0.4	4.1 ± 0.2	4.8 ± 1.3	4.2 ± 0.4
Total	100	100	100	100

[a]The peaks are constituted by 39 compounds identified in Table 2. The percentage of each peak was compared between the groups of workers with an ANOVA (post-hoc comparison: Tukey HSD test for unequal sample size). Categories differing at $P < 0.05$ are indicated with different letters. Two workers switched groups between successive measurements.

and sterile workers, respectively (mean ± SE, i.e., 7.6 times more on alphas), while pentane extracted 33 ± 11 vs. 5 ± 2 μm on alphas and sterile workers, respectively (6.6 times more on alphas).

DISCUSSION

Gas chromatography and mass spectrometry of long-chain hydrocarbons on the cuticle clearly demonstrate that the alpha workers of *D. quadriceps* differ from their sterile nestmates by the relative proportion of 9-hentriacontene. This difference was not affected by colony membership since it was found for all alphas. Discriminant analysis revealed that 9-tritriacontene is also important to discriminate between groups. It differed significantly between young workers and virgin alphas, and its proportion was lowest in old sterile workers, suggesting that it is characteristic of the cuticle of recently eclosed workers.

The alpha ant lays most or all the eggs in *D. quadriceps* and is the only

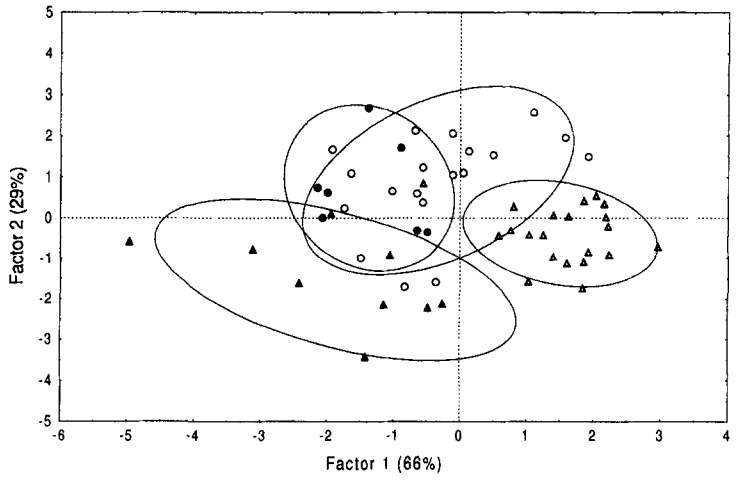

FIG. 5. Same discriminant analysis after removing 9-hentriacontene and 9-tritriacontene. Areas were restandardized with the geometric mean of the 12 remaining peaks. The groups are no longer separated.

individual with fully developed ovaries. Although one to three high-ranking workers sometimes lay a few eggs, their ovaries are much less developed (Monnin and Peeters, 1997). The correlations presented here strongly suggest that 9-C_{31} levels are related to ovarian activity. Virgin and mated alphas had significantly different levels, which may be associated with a small difference in ovarian activity due to insemination. Cuticular hydrocarbons have been shown to transmit information used in colonial or kin recognition in a number of ants, bees, and wasps (Bonavita-Cougourdan et al., 1987; Espelie et al., 1994; Arnold et al., 1996; Bagnères et al., 1996). We think this function may also be important in *D. quadriceps*, and thus the differences in long-chain hydrocarbons described in our study are probably superimposed onto intercolony differences.

Repeated SPME-GC measurements of the same individuals demonstrated a low variability, and thus this extraction technique can produce reliable data about individuals. Although SPME slightly altered the profile of a reference mixture, its results are consistent when extracting both this mixture as well as cuticular hydrocarbons from live ants, thus allowing the comparison of individuals. SPME presents several advantages over classical solvent extraction. It is easy to use and, most of all, it is not destructive and allows the study of valuable individuals without sacrificing them. Thus, it also permits studying of the tem-

poral dynamics of an hydrocarbon profile. Since it is solventless, it does not extract compounds from inside the insect, unlike whole-body solvent washes. Furthermore, SPME permits extraction of a precise part of the body. Given the large size of *D. quadriceps* workers, a sufficient quantity of cuticular hydrocarbons could be extracted that allowed GC analysis, and even GC-MS. This may be more difficult with smaller insects, although sensitivity is increased since no solvent is used. Nevertheless, SPME has the disadvantage that samples cannot be stored and must be analyzed immediately.

Long-chain hydrocarbons were more abundant on the abdomen than on the head and petiole, suggesting that they are synthesized in this part of the body. 9-C_{31} was not found in the Dufour's glands of two alphas, whereas it had been measured on their cuticle. Since the antennae of subordinates are rubbed against the membranes between abdominal segments V and VII, which lie over the pygidial gland in *D. quadriceps* (J. Billen, personal communication), this gland needs to be considered as a possible source of 9-C_{31}. In *D. australis* the pygidial gland is composed of secretory cells connected to a small invagination of the intersegmental membrane, which forms a tiny reservoir (Billen et al., 1995). We initially dissected the pygidial glands of alphas and sterile workers and extracted them in solvent, but failed to obtain resolution of any peaks. Comparative insect physiology hints that 9-C_{31} and other long-chain hydrocarbons are more likely to be produced by the oenocytes, which lie just underneath the intersegmental membranes of the abdomen. Epidermal oenocytes are located at the base of the epidermis, usually against the basal lamina, and are generally involved in the biosynthesis of cuticular hydrocarbons. Indeed, oenocytes may be considered to be true exocrine cells, because their secretory products are transported to the cuticle (Noirot and Quennedey, 1991). In honeybees, the oenocytes secrete wax, which is carried to the outside via microtubules (Hepburn, 1986). In the desert locust, oenocytes synthesize cuticular lipids (Diehl, 1975), and in *Culicoides nubeculosus* (Diptera), they are involved in the production of sex pheromones present on the cuticle (Ismail and Zachary, 1984). A relationship between ovarian maturation and sexual attractiveness (mediated by cuticular hydrocarbons) has been shown in *Calliphora vomitoria* (Diptera) (Trabalon et al., 1990). Whatever their precise glandular origin in *D. quadriceps*, long-chain hydrocarbons seem to diffuse over the rest of the body surface. Rubbing the antennae of subordinates on the alpha's abdomen may be an efficient means to transfer nonvolatile cuticular hydrocarbons (the abdomen yields the highest amount of hydrocarbons).

Dinoponera australis is another species in which only the alpha worker can mate and has active ovaries (Paiva and Brandão, 1995). After studying a single colony, Oldham et al. (1994) reported that the alpha differed from sterile workers in her mandibular gland secretions (aldehydes and pyrazines). However this difference concerns the total amount of secretion (one gamergate was extracted

once in solvent). Oldham et al. (1994) suggested that this difference is linked either with reproduction or with foraging. The latter seems better supported by the limited evidence, since the mandibular gland of one forager contained the highest amount of secretion, while the gland of the gamergate contained the lowest.

Most polistine wasps lack a morphologically specialized reproductive caste, and sterility is regulated through aggressive interactions—only the alpha lays eggs, as in many queenless ants (Reeve, 1991; Röseler, 1991a). In *P. dominulus*, the alpha was found to differ in the relative proportions of several hydrocarbons (C_{31}–C_{35}) relative to her sterile subordinates (Bonavita-Cougourdan et al., 1991). The characteristic cuticular hydrocarbon profile of the alpha ant was not colony-specific, and the alpha differed more from her subordinates than these differed from foreign workers. Several cuticular compounds identified in *P. dominulus* were the same as in *D. quadriceps*.

In bumblebees (*Bombus hypnorum*), reproductive division of labor is also regulated by a dominance hierarchy (which can be affected by size differences among the monomorphic adult females) (Röseler, 1991b). The alpha individual lays most of the eggs, although high-ranking subordinates also oviposit. Pentane rinses of the cuticle yielded a variety of long-chain alkanes, alkenes, and alkadienes (Ayasse et al., 1995). Alphas had higher amounts of (*Z*)-11-pentacosene, (*Z*)-7-pentacosene, methyltricosene, methylpentacosene, heptacosadiene, octacosadiene, and tricontadiene relative to egg-laying subordinates, which themselves yielded higher amounts than sterile subordinates. These differences were found in all colonies, and the alphas differed more from their nestmate workers than the latter differed from foreign workers (Ayasse et al., 1995).

The queenless ant *D. quadriceps*, the wasp *P. dominulus*, and the bee *B. hypnorum* share the characteristic that reproductive differentiation occurs in the adult stage (unlike social insects with dimorphic queens and workers). Dominance interactions regulate ovarian activity, and there is accumulating evidence that differences in cuticular hydrocarbons characterize fertile and sterile individuals. Other studies have shown that either dominance rank or ovarian development is recognized by means other than physical aggression alone (West-Eberhard, 1977; Downing and Jeanne, 1985). Similarly in honeybees, orphaned workers with active ovaries are discriminated against (Visscher and Dukas, 1995). Unlike the more complex differences in hydrocarbon bouquets described in *P. dominulus* and *B. hypnorum*, the olfactory signal of fertile workers in *D. quadriceps* appears to differ by one hydrocarbon only, revealing it to be an ideal species to investigate status recognition. Bioassays are now needed to test the activity of 9-hentriacontene. The nondestructive nature of SPME has also enabled us to study the temporal characteristics of the induction of 9-C_{31} in workers that accede to the alpha rank (Peeters et al., unpublished data).

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SEX PHEROMONE COMPONENTS OF PITCH PINE LOOPER, *Lambdina pellucidaria*

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Abstract—Two methylated hydrocarbons, 7-methylheptadecane (7) and 7,11-dimethylheptadecane (7,11), are sex pheromone components of female pitch pine looper (PPL), *Lambdina pellucidaria*. Compounds extracted from the pheromone glands of female moths were identified by coupled gas chromatographic-electroantennographic detection (GC-EAD) and coupled GC-mass spectrometry (GC-MS) in selected ion monitoring mode. In field-trapping experiments, 7 and 7,11 in combination, but not singly, attracted numerous male moths. 5,11-Dimethylheptadecane (5,11) was detected by GC-EAD in female PPL pheromone gland extract, but did not significantly increase attraction of PPL males to 7 plus 7,11. Although 7 was >10 times more abundant than 7,11 in pheromone gland extracts, traps baited with synthetic 7 plus 7,11 at a blend ratio of 1:1, rather than 1:0.1 or 1:0.01, captured the most PPL males. The chemical communication of PPL and spring hemlock looper (SHL), *Lambdina athasaria*, is strikingly similar. Both species employ 7 plus 7,11 as sex pheromone. Restriction of SHL to forests with eastern hemlock or balsam fir and PPL to forests with pitch or other hard pines contributes to their reproductive isolation. PPL and SHL may also use different optical isomers of enantiomeric 7 and stereoisomeric 7,11 to maintain specificity of their chemical communication.

Key Words—Lepidoptera, Geometridae, *Lambdina athasaria*, *Lambdina fiscellaria*, *Lambdina pellucidaria*, sex pheromone, synergism, 7,11-dimethylheptadecane, 5,11-dimethylheptadecane, 7-methylheptadecane.

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INTRODUCTION

In northeastern North America, the univoltine pitch pine looper moth (PPL), *Lambdina pellucidaria* (Grote and Robinson) (Lepidoptera: Geometridae), has sporadic outbreaks during which the larvae damage pitch pine, *Pinus rigida* Miller, and to a lesser extent, red pine, *P. resinosa* Aiton, shortleaf pine, *P. echinata* Miller, and Virginia pine, *P. virginiana* Miller (Craighead, 1950; Sorensen and Barbosa, 1975; Maier, unpublished data). During the last outbreak, in the early 1990s, PPL defoliated nearly 400,000 acres of pitch pine in the Pine Barrens of New Jersey and additional acreage in coastal Massachusetts and on Long Island, New York (USDA, 1992, 1994). By increasing needle drop and thus dead matter beneath trees, PPL increases the risk of fire in outbreak areas. Like the spring hemlock looper (SHL), *L. athasaria* (Walker) (Maier and Lemmon, 1996), PPL overwinters as a pupa in the duff beneath host trees. By contrast, the eastern hemlock looper (EHL), *L. fiscellaria fiscellaria* (Guenée), and the western hemlock looper (WHL), *L. fiscellaria lugubrosa* (Hulst), overwinter as eggs. Adults of PPL and SHL fly in spring, whereas those of EHL and WHL fly in late summer and early autumn.

Females of SHL, EHL, and WHL attract mates by releasing pheromones, consisting of mono- and/or dimethylheptadecanes (Gries et al., 1991, 1993, 1994; Li et al., 1993a,b). Identification of the sex pheromone produced by congeneric PPL would provide an opportunity to develop a monitoring system for this destructive pest. We report the identification and field testing of sex pheromone components of PPL.

METHODS AND MATERIALS

Laboratory Analyses. Pupae were collected in the duff under pitch pines at Myles Standish State Forest, Plymouth County, Massachusetts, during April 1994. Chilled pupae were sent to Simon Fraser University, placed into filter paper-lined Petri dishes and gradually warmed (2–22°C at ~1°C/day) over a period of 20 days under a 14L:10D photoperiod. Emergent moths were separated to avoid mating. Abdominal tips with pheromone glands of 1- to 2-day-old calling virgin females were removed and extracted in hexane for 5 min. Aliquots of one female equivalent (1 FE) of pheromone extract were subjected to gas chromatographic analyses with both flame ionization and electroantennographic detection (GC-EAD) (Arn et al., 1975) on three fused silica columns [DB-5, DB-210 (30 m × 0.25 mm ID), and DB-23 (30 m × 0.32 mm ID); J&W Scientific, Folsom, California 95630].

Coupled GC-mass spectrometry (MS) (Hewlett Packard 5985B, fitted with a DB-23 coated column) in full-scan and selected ion monitoring (SIM) mode

was conducted to confirm the presence of candidate pheromone components in gland extracts. For GC-MS-SIM, full-scan electron impact spectra of synthetic compounds were obtained to select ions indicative of methyl branch positions (Pomonis et al., 1980; Gries et al., 1994). In sequence, 200 pg of synthetic compounds, a hexane blank, and a concentrated pheromone gland extract (30 FE) were then chromatographed, scanning for the diagnostic ions.

Synthesis. Candidate pheromone components 7-methylheptadecane (7), 7,11-dimethylheptadecane (7,11), and 5,11-dimethylheptadecane (5,11), were available from previous studies (Gries et al. 1991, 1994). Each of enantiomeric 7, stereoisomeric 7,11, and stereoisomeric 5,11 was >95% chemically pure. None of the chemical contaminants elicited antennal responses in GC-EAD recordings.

Field Bioassay. Field experiments were conducted at Myles Standish State Forest (Plymouth, Plymouth County, Massachusetts) in an area with about 40% pitch pine and 60% scrub oak, *Quercus ilicifolia* Wang. Experiments were set up in randomized, complete blocks with traps and blocks at 50-m intervals. Green Unitraps (Phero Tech Inc., Delta, British Columbia, V4G 1E9) were suspended from pitch pine trees at 1.5–2 m above ground and were baited with rubber septa (The West Company, Lionville, Pennsylvania 19341) impregnated with candidate pheromone components in HPLC-grade hexane, hexane, or baited with virgin females of PPL. Virgin females 1 to 2 days old were confined singly in 30 × 30 × 25-mm (high) wire mesh cages placed below the lid of Unitraps or suspended from the inside top of Pherocon wing traps (Trécé Inc., Salinas, California 93912). Each moth was provided with a 10% sucrose solution in a 9 × 30-mm shell vial with a cotton wick. A Dichlorvos (Bio-Strip, Inc., Reno, Nevada 89507) cube was placed in the bottom of each Unitrap to ensure rapid death of captured moths and potential insect predators. Wing traps had no Dichlorvos. Captures of males in baited traps were compared in three experiments. Experiment 1 (May 27–31, 1994) tested candidate pheromone components 7; 7,11; and 5,11 alone and in ternary combination. Experiment 2 (June 8–9, 1994) tested virgin females in Unitraps and wing traps and synthetic 7; 7,11; and 5,11 in all binary and ternary combinations. Experiment 3 (June 21–22, 1994) tested 7 at 100 μg in binary and ternary combination with 7,11 and 5,11 each at 100, 10, or 1 μg. Each experiment was replicated 10 times. Voucher specimens of PPL are deposited in the insect collection in the Department of Entomology, Connecticut Agricultural Experiment Station, New Haven.

Statistical Analyses. Despite transformation, data of all three field experiments were not normally distributed and were therefore subjected to nonparametric analyses of variance by ranks (Friedman's test) followed by the Newman-Keuls test (Zar, 1984; SAS/STAT User's Guide, 1988, release 6.03 edition, SAS Institute, Inc., Cary, North Carolina 27513).

RESULTS

Laboratory Analyses. GC-EAD analyses of female PPL pheromone gland extract revealed three EAD-active compounds (Figure 1). DB-23, DB-210, and DB-5 column retention indices of compounds **1** (~100 pg/FE), **2** (<10 pg/FE), and **3** (<10 pg/FE) were identical to those of 7; 7,11; and 5,11, respectively, which were previously identified in SHL (Gries et al., 1994). Equivalent amounts of SHL-produced and synthetic 7; 7,11; and 5,11 elicited comparable antennal responses by male PPL in GC-EAD recordings.

GC-MS-SIM analyses of pheromone extract and synthetic candidate pher-

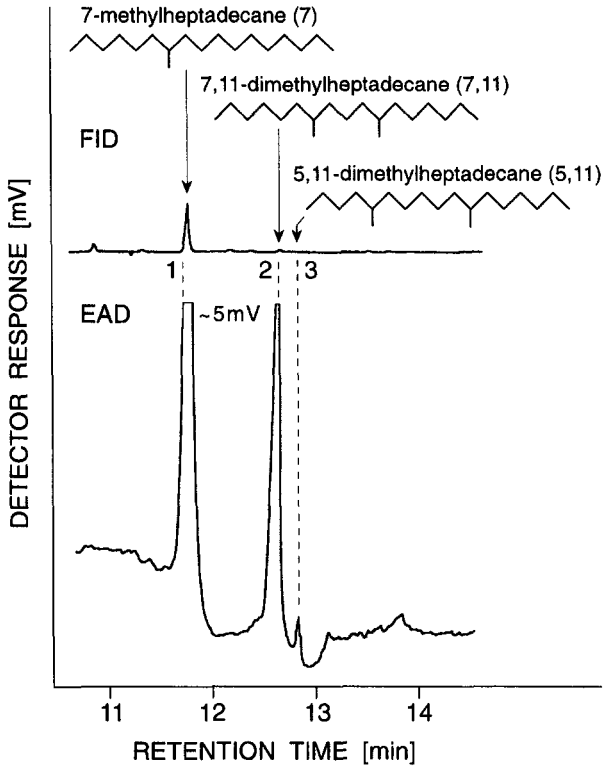

FIG. 1. Representative recording (N = 8) of flame ionization detector (FID) and electroantennographic detector (EAD: male moth antenna) responses to one female equivalent of female PPL pheromone gland extract. Antennal response to 5,11 was small but reproducible. Chromatography: splitless injection; injector and FID temperature: 240°C; DB-210 column; 1 min at 50°C, 20°C/min to 130°C, 2°C/min to 180°C, then 10°C/min to 220°C.

omone components, scanning for three ions indicative of methyl branch positions in 7; 7,11; and 5,11, resulted in ion ratio and retention time matches of synthetic versus female-produced 7 and 7,11. GC-MS-SIM, *m/z* (relative intensity)—synthetic 7: 112 (100), 168 (42), 169 (31); in gland extract: 112 (100), 168 (46), 169 (32); synthetic 7,11: 112 (100), 155 (23), 183 (25); in gland extract: 112 (100), 155 (24), 183 (23). Fragmentation ions indicative of 5,11 were below detection threshold.

Field Bioassay. Unitraps baited with the ternary blend of enantiomeric 7, stereoisomeric 7,11, and stereoisomeric 5,11 captured numerous males of PPL, whereas none of the compounds tested alone were very attractive (Figure 2, experiment 1). Blends of 7 plus 7,11 and of 7; 7,11; and 5,11 were equally attractive, and more attractive than 7 plus 5,11 (Figure 3, experiment 2). Numbers of PPL males captured in traps baited with virgin females or with 7,11 plus 5,11 did not significantly differ from the numbers captured in unbaited control

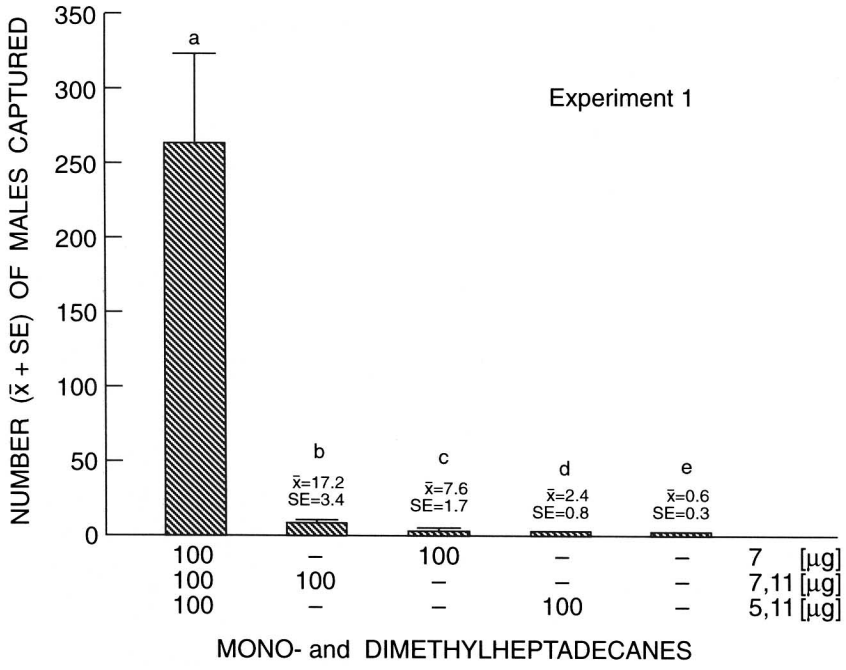

FIG. 2. Captures of male *L. pellucidaria* in Unitraps baited with candidate pheromone components alone at 100 µg and in ternary combination at 100 µg each. Myles Standish State Forest, Plymouth, Massachusetts, May 27–31, 1994; 10 replicates. Bars with the same letter superscript are not significantly different, $P < 0.05$.

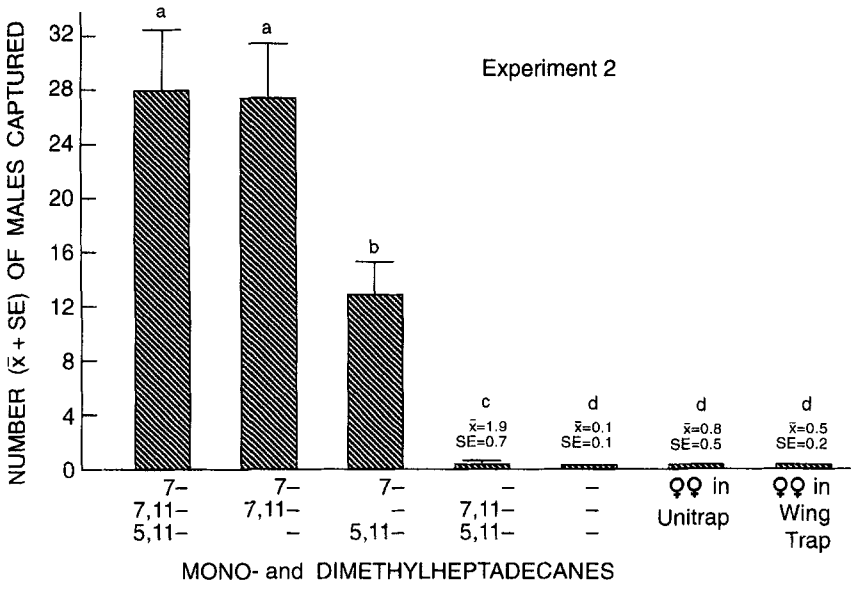

FIG. 3. Captures of male *L. pellucidaria* in Unitraps baited with candidate pheromone components in ternary and binary combinations at 100 μg each or with virgin females, and in Pherocon wing traps baited with virgin females. Myles Standish State Forest, Plymouth, Massachusetts, June 8–9, 1994; 10 replicates. Bars with the same letter superscript are not significantly different, $P < 0.05$.

traps. Decreasing proportions of 7,11 in a binary blend (7 plus 7,11) and ternary blend (7; 7,11; and 5,11) resulted in decreasing numbers of captured PPL males (Figure 4, experiment 3). In all experiments, only PPL males were captured.

DISCUSSION

Evidence that 7 and 7,11 are sex pheromone components of PPL includes: (1) EAD-active compounds **1** and **2** in female PPL pheromone gland extracts (Figure 1) had the same retention characteristics as synthetic 7 and 7,11, respectively, on fused silica columns coated with DB-5, DB-210, and DB-23; (2) GC-MS analyses in SIM mode of female PPL-produced **1** and synthetic 7, and PPL-produced **2** and synthetic 7,11, resulted in identical retention characteristics and excellent ion ratio matches of female-produced and synthetic compounds; (3) PPL-produced and synthetic compounds, at equivalent quantities, elicited comparable antennal responses in GC-EAD recordings; and (4) in field experiments, rubber septa impregnated with 7 plus 7,11 attracted numerous male PPL moths.

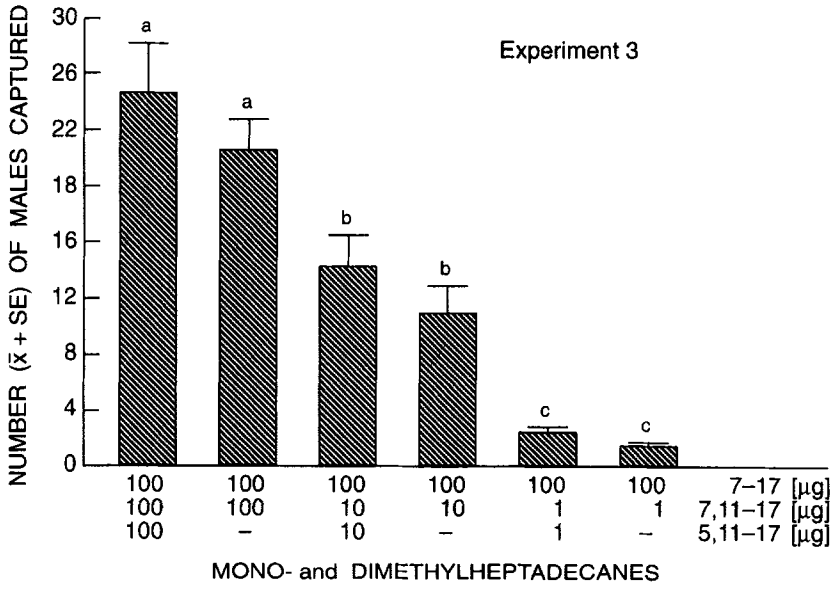

FIG. 4. Captures of male *L. pellucidaria* in Unitraps baited with 7; 7,11; and 5,11 at different blend ratios. Myles Standish State Forest, Plymouth, Massachusetts, June 21–22, 1994; 10 replicates. Bars with the same letter superscript are not significantly different, $P < 0.05$.

Greatest abundance and EAD activity of 7 in pheromone gland extracts (Figure 1) suggested, and field experiment 2 confirmed (Figure 3), that 7 is an essential component of the PPL sex pheromone. Indeed, lures without 7 failed to attract significant numbers of PPL males (Figures 2 and 3). Because synergistic behavioral activity of 7,11 exceeded that of 5,11, and 5,11 failed to enhance significantly the attractiveness of 7 plus 7,11, 5,11 appears to be least important in, or may not even be a component of, the PPL sex pheromone blend.

If we were to hypothesize that female PPL release 7 and 7,11 at the same wide ratio as found in pheromone glands (Figure 1), the greatest attractiveness of synthetic pheromone at a 1:1 ratio (Figure 4) is surprising but consistent with findings in WHL. In gland extracts of female WHL, the major pheromone component 5,11 is more abundant than synergistic 7 and 2,5 (Gries et al., 1993), but a 1:1:1 ratio of 5,11; 7; and 2,5 is most attractive to males seeking mates (Krannitz, 1992). If ratios of components in gland extracts and in effluvia of calling females were similar, then the strong attraction of PPL and WHL males seems to be mediated by absolute abundance, more than "proper" ratio, of pheromone components. Optimal attraction of many other (tortricid) moths, in

contrast, requires a pheromone lure that mimics the blend ratio of pheromone components found in pheromone glands or effluvia of calling females (Arn et al., 1992).

Chemical (sexual) communication in the PPL and in congeneric SHL, WHL, and EHL is strikingly similar. Females of all four congeners employ

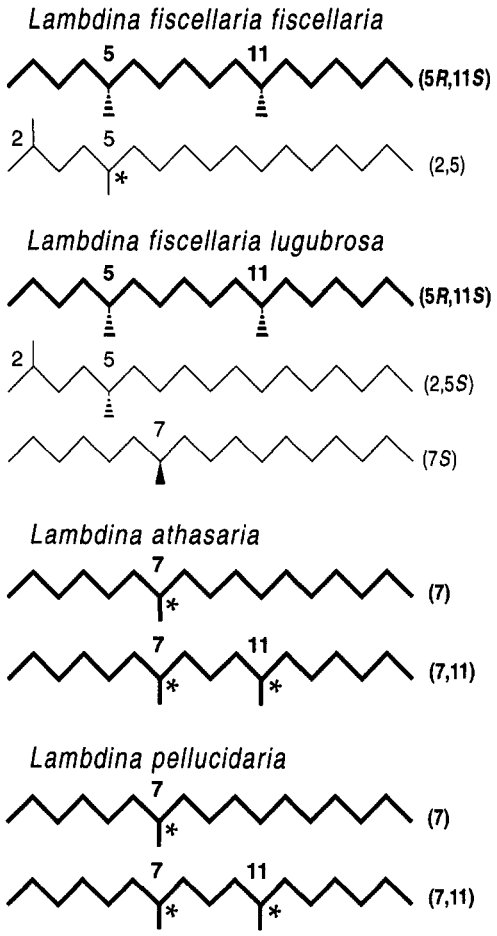

FIG. 5. Pheromone components of eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria* (Gries et al., 1991; Li et al., 1993a); western hemlock looper (WHL), *L. fiscellaria lugubrosa* (Gries et al., 1993; Li et al., 1993b); spring hemlock looper (SHL), *L. athasaria* (Gries et al., 1994); and pitch pine looper (PPL), *L. pellucidaria* (this paper). Boldface molecules are essential for attraction of males. *Absolute configuration of pheromone component not yet determined.

mono- and/or dimethylheptadecanes to attract conspecific males (Figure 5). Moreover, all *Lambdina* females tested in trapping experiments were only weakly attractive to conspecific males (Otvos, 1972; Ostaff et al., 1974; Gries et al., 1994). Although the caging of females in these experiments may have interfered with their characteristic calling and mating behavior (West and Bowers, 1994), low pheromone titers in gland extracts (Figure 1; Gries et al., 1991, 1993, 1994) suggest that *Lambdina* females indeed release little pheromone.

Resemblance of their chemical communication supports the generic placement of PPL, SHL, EHL, and WHL. Among *Lambdina* spp. associated with hemlock, *Tsuga* spp., spatial separation of coseasonal EHL and WHL, temporal separation of sympatric EHL and SHL, and specificity of pheromonal blends effectively prevent interspecific matings. PPL and SHL are coseasonal and both use 7 plus 7,11 as sex pheromone, but several factors may contribute to their reproductive isolation: (1) PPL and SHL differ in appearance (Capps, 1943; Forbes, 1948). The ground color of wings is uniformly mouse gray in PPL and is cream-colored and conspicuously speckled with gray in SHL (Forbes, 1948). (2) PPL and SHL are mostly allopatric in eastern North America. PPL occurs in dry sandy areas with mainly pitch and other hard pines. SHL, in contrast, typically inhabits moist areas with forests of eastern hemlock, *Tsuga canadensis* (L.) Carrière, or balsam fir, *Abies balsamea* (L.) Miller. (3) Finally, females of PPL and SHL may use different enantiomers of 7 and/or stereoisomers of 7,11 to attract conspecific males. This hypothesis is currently being tested.

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INTERACTIONS AMONG INSECT HERBIVORE GUILDS: INFLUENCE OF THRIPS BUD INJURY ON FOLIAR CHEMISTRY AND SUITABILITY TO GYPSY MOTHS

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Abstract—This study investigated the consequences of early season bud herbivory on host-plant phytochemistry and subsequent effects on a later mid-season leaf-feeding herbivore, to test the hypothesis that temporally segregated interguild interactions could affect herbivore success through plant-mediated responses. Our system consisted of American basswood, *Tilia americana*, a bud-feeding thrips species, *Thrips calcaratus*, and the folivorous gypsy moth, *Lymantria dispar*. The impact of thrips bud-feeding on American basswood foliar chemistry and subsequent effects on gypsy moth larval preference and performance were measured. Foliar total nonstructural carbohydrates increased and phenolic levels decreased in response to bud injury, which affected larval feeding preference. In a two-choice test, gypsy moth larvae preferred leaf discs with high carbohydrate and low phenolic levels. The effects on larval performance depended on the extent of prior bud injury and were correlated with carbohydrate concentrations. In an early season assay, larval performance was lowest on moderately bud-damaged tissue, which also had the lowest total nonstructural carbohydrates. In a mid-season assay, larval performance and carbohydrate concentrations were highest in severely bud-damaged foliage. Foliar phenolics were highest in severely bud-damaged tissue in the early season assay, and in moderately damaged tissue in the mid-season assay. Gypsy moth performance was not correlated with foliar phenolic levels. Secondary (reflushed) foliage had higher carbohydrate levels than did primary (original) foliage, which correlated with increased larval performance. This study illustrates that bud-feeding herbivores can alter the phytochemistry and subsequent suitability of host-plant foliage for later folivores. The implications of these results to interactions between feeding guilds, community structure, and forest health are discussed.

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Key Words—Phytochemistry, herbivory, feeding guilds, bud injury, thrips, *Tilia*, plant-insect interactions, gypsy moth, *Lymantria dispar*, host preference.

INTRODUCTION

Prior herbivory can affect the acceptability and suitability of a host plant to subsequent herbivores (Rhoades, 1985; Tallamy and Raupp, 1991; Karban and Niiho, 1995; Baldwin and Schmelz, 1996). Both rapid and delayed responses have been shown in a variety of plant-herbivore systems, including herbaceous plants (Kogan and Fischer, 1991; Tallamy and McCloud, 1991), deciduous trees (Mattson and Palmer, 1988; Wainhouse et al., 1988; Clausen et al., 1991; Faeth, 1991; Robison and Raffa, 1994), and conifers (Wagner, 1988; Raffa, 1991; Krause and Raffa, 1995). The effects and mechanisms of induced plant responses may be complex (Wagner and Evans, 1985; Bultman and Ganey, 1995; Bauce and Carisey, 1996) and are probably system-dependant. For example, prior herbivory can reduce subsequent herbivore success by decreasing foliar nutrient quality or eliciting allelochemical production. In contrast, prior defoliation can sometimes improve herbivore performance by eliciting the accumulation of foliar nutrients or depleting foliar defensive compounds. An enhanced nutritional substrate could also ameliorate the effects of induced plant defenses, resulting in "induced amelioration" as described by Haukioja et al. (1990). Rhoades (1985) suggests that in a given plant, several of these processes can occur simultaneously.

Temporally segregated relationships become increasingly complex when interactions among different feeding guilds are considered. There is a substantial knowledge base on the effects of foliar damage on subsequent folivore success (Haukioja and Niemela, 1979; Bergelson et al., 1986; Fowler and McGarvin, 1986; Hartley and Lawton, 1987; Hunter, 1987; Neuvonen et al., 1988). However, relatively few studies have considered host-mediated interactions across feeding guilds (but see Danell and Huss-Danell, 1985; Faeth, 1986, 1992a, b; Martin et al., 1994). Under natural conditions, such interactions are common (Schowalter, 1986). Temporally segregated interactions can occur across existing plant parts, such as when foliar- or root-feeding insects increase susceptibility to subsequent colonization by stem colonizing bark beetles (Wright et al., 1979; Klepzig et al., 1991), or they can be due to plant phenology (Faeth, 1986; Martin et al., 1994).

Bud feeding has been shown to alter plant architecture and physiology (Whitham and Mopper, 1985; Haukioja et al., 1990) and phytochemistry (Mattson and Koller, 1983; Clancy, 1991). However, data on the quality of subsequent foliage, as well as any impacts on subsequent folivores, are lacking. Our study considered the effects of prior bud-feeding on subsequent herbivory among

two insect species that share a common deciduous host plant. In particular, we considered whether the occurrence and extent of bud feeding by a rasping/sucking thrips affects the behavior, physiology, and chemical environment of a leaf chewing caterpillar.

The introduced basswood thrips, *Thrips calcaratus* Uzel (Thysanoptera: Thripidae), is a common bud-feeding insect in Wisconsin. This species is univoltine, and sometimes undergoes widespread population eruptions that have regional impacts (Raffa et al., 1992; Rieske et al., 1992; Rieske, 1995). It is a parthenogenetic feeding specialist on American basswood, *Tilia americana* L., which is a major component of northern hardwood forests in the eastern United States. Adults emerge from overwintering sites and feed on expanding buds in early to mid-May in northern Wisconsin (42°30′N, 80°00′W). Damage is characterized by a stunted, shredded appearance of expanding leaves. Dense populations cause phytohormone imbalances that can induce bud or premature leaf abscission (Rieske and Raffa, 1995). Repeated defoliation causes branch dieback, thinned crowns, and reduced radial growth (Raffa et al., 1992; Rieske et al., 1992).

American basswood is also a preferred host for larvae of the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) (Drooz, 1985; Montgomery and Wallner, 1988), which feed and develop on expanding foliage. This univoltine folivore is a generalist feeder with an extremely wide host range (Drooz, 1985). Both the introduced basswood thrips and the gypsy moth are introduced herbivores subject to periodic outbreaks.

The purpose of this study was to assess the impact and extent of herbivory by a specialist bud feeder on the subsequent success of a generalist foliage feeder. Specifically, the objectives were to: (1) assess the preference of gypsy moth larvae for foliage from bud-damaged versus undamaged trees, (2) assess the effects of foliage from bud-damaged trees on gypsy moth growth and development, (3) quantify the effects of thrips bud-feeding on foliar total nonstructural carbohydrates and foliar phenolics, and (4) relate potential variations in gypsy moth larval preference and performance to changes in foliar chemistry.

The issue of how best to study plant responses to herbivory is itself the subject of much debate (Neuvonen and Haukioja, 1985; Karban and Myers, 1989; Haukioja, 1991). It is widely recognized that applying controlled injury to test plants provides experimental manipulations that can be more readily interpreted as causality, but likewise that such studies are somewhat unrealistic in that they use artificial means of injury; artificial conditions caused by caging, tissue removal, or other manipulations; or plant stages and phenologies selected by the investigator rather than the herbivore (Neuvonen and Haukioja, 1985; Jones and Coleman, 1988; Krause and Raffa, 1992). Conversely, field studies in which herbivory occurs naturally and plants from various injury classes are chemically analyzed or subjected to bioassays (Whitham and Mopper, 1985;

Neuvonen et al., 1988; Klepzig et al., 1996) provide a more realistic interaction, but cannot totally remove the possibility of spurious correlations. In order to reconcile these trade-offs, we have used both approaches. In a previous study, we reported on the effects of controlled feeding by the introduced basswood thrips on American basswood seedling phytochemistry (Rieske and Raffa, 1995). In the present study, we sampled naturally infested large trees in the field and evaluated foliar chemistry and suitability to a subsequent herbivore.

METHODS AND MATERIALS

American basswood foliage showing various levels of bud-feeding damage was assayed for gypsy moth larval suitability and analyzed for differences in levels of foliar carbohydrates and phenolics. Foliage was collected on June 28 and July 12, representing "early season" and "mid-season" collection dates, from sites in northeast Wisconsin (Forest County) with a history of damage by the introduced basswood thrips. Individual trees were visually classified as having "low" (barely detectable), "moderate," or "severe" defoliation (Bosshard, 1986). These levels corresponded to 0–20%, 21–69%, and >70% of the canopy affected by thrips feeding, and they were chosen because thrips defoliation was easily categorized into these levels by three independent observers. Twelve trees were haphazardly chosen in each of the three damage classes. Foliage was randomly collected from damaged trees at a height of $2\frac{1}{2}$–3 m and returned to the laboratory for bioassays and phytochemical analysis. The second (mid-season) collection contained both primary (original) and secondary (reflushed) foliage in the severe damage class due to the indeterminant growth characteristic of American basswood. Primary and secondary foliage were treated separately in all assays.

Prior to use in assays, gypsy moth larvae were reared on wheat-germ-based artificial diet in growth chambers with a 15L:9D photoperiod at 23°C.

Preference. The preference of second instars of the gypsy moth was measured with leaf discs in a two-choice test. Two 15-mm-diameter leaf discs were placed adjacent to one another near the center of a 5.5-cm-diameter Petri dish. The discs were anchored to a paraffin wax bottom by small pins passed through Whatman No. 1 filter paper moistened with distilled water.

Each individual disc was classified as either "bud-damaged," originating from trees with >70% of the canopy affected by thrips bud feeding, or "control," originating from trees with 0–20% of the canopy damaged by thrips bud feeding. Control trees were randomly paired with bud-damaged trees. There were five replicates of each pairing, for a total of 60 two-choice tests on each assay date. Severely thrips-damaged leaf discs consisted exclusively of second-

ary tissue for the second assay. One larva per assay arena ($N = 60$) was allowed to feed for 24 hr at 23°C with a 15L:9D photoperiod. Assays were monitored at 4-hr intervals and were terminated at 8 hr or when there was a difference greater than 50% in the area consumed between treatments. Feeding was quantified by digitized images with a Macintosh computer and scanner.

Performance. Performance was assessed by allowing second instars to feed for the duration of the instar on excised leaves of trees from the low (0–20% of the buds thrips-damaged), moderate (21–69%), or severe (>70%) damage classes. Performance assays were initiated with newly molted second instars and terminated when larvae molted to third instars. There were three replicates from each of the 36 trees (12 per damage class) for a total of 108 assays. Whole leaves were weighed and placed in florists' water picks in 21.5 × 7 × 5.5-cm clear plastic rearing boxes. One larva was placed in each assay arena and monitored at approximately 24-hr intervals for the duration of the assay. At each monitoring interval, larvae and fresh leaves were weighed and the leaves were replaced. Performance assays were maintained under the same temperature and photoperiod as the preference assays and were terminated when the insect molted. At the completion of the assay, all plant tissue and larval cadavers were oven dried (65°C for seven days) and weighed. Relative growth rate [RGR = larval biomass gained (milligrams)/initial larval dry wt (milligrams)/time (days)], relative consumption rate [RCR = leaf tissue consumed (milligrams)/initial larval dry wt (milligrams)/time (days)], and length of the second stadium (days between larval molts) were calculated for each assay date as measures of insect performance.

Foliar Total Nonstructural Carbohydrate Analysis. Methods were adapted from Quarmby and Allen (1989), and American basswood tissue from each of the three damage classes was ground, extracted, and analyzed spectrophotometrically for total nonstructural carbohydrates. Air dried tissue (50 mg) was extracted for 2 hr in 30 ml of distilled water in a reciprocating hot water (97°C) bath. Extracts were filtered through Whatman No. 1 filter paper and diluted to 50 ml. In a darkened ice bath, 0.5 ml extract was added to 2.5 ml anthrone reagent, consisting of sulfuric acid, anthrone (Sigma Chemical Company, St. Louis, Missouri), and thiourea (thiocarbamide; Sigma). Samples were placed in a boiling water bath in a darkened hood for 10 min, then transferred to an ice bath and allowed to cool. A Microplate Autoreader EL311 (Bio-Tek Instruments, Winooski, Vermont) was used to read absorbance at 630 nm with sucrose as a standard.

Foliar Phenolics Analysis. Total foliar phenolics from each damage class were determined with Folin and Ciocalteu's phenol reagent (Sigma), from methods adapted from Kelsey and Harmon (1989). Air-dried tissue was extracted in 5 ml 80% acetone for 16 hr at 23°C. A 200-μl aliquot of extract was diluted

with 5 ml distilled water. One milliliter of Folin–Ciocalteu reagent was added, followed by 5 ml 20% Na_2CO_3. Following an incubation period of 2 hr, absorbance at 630 nm was read and compared to a tannic acid standard.

Foliar Fiber and Nutrient Analysis. Dried plant tissue from each damage class was analyzed by the University of Wisconsin Soil and Plant Analysis Laboratory for thrips-induced changes in foliar acid-digested fiber and nutrients, including nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, zinc, boron, manganese, iron, copper, aluminum, and sodium.

Statistical Analyses. A paired *t* test was used to analyze larval feeding preferences between the bud-damaged and control discs. Differences in larval performance among levels of bud herbivory were assessed by analysis of variance. When necessary, a log transformation was used to normalize data prior to analysis.

Least-squares means was used to analyze temporal changes in foliar total nonstructural carbohydrates, phenolics, fiber, and nutrients. Analysis of variance was used to analyze differences based on bud injury, with log transformation when necessary. Differences were determined by Fisher's Protected LSD (Abacus Concepts, 1989), with $P \leq 0.08$ considered significant. Linear regression analysis was used to determine the relationship between foliar total nonstructural carbohydrates, phenolics, fiber, and nutrients and the gypsy moth performance parameters.

RESULTS

Preference. In the preference assay with early season foliage, gypsy moth larvae preferred control discs over thrips-damaged discs (Figure 1a). However, in the assay with mid-season foliage (Figure 1b), which compared secondary damaged tissue to primary control tissue, this preference was reversed, and larvae showed a strong preference for the bud-damaged discs. Figure 1c provides the pooled data from both assays; the net result of intense bud feeding was an increase in larval preference for the host plant.

Performance. Table 1 shows the relative growth rates (RGRs) of larvae reared on each of the three bud damage classes for the early season assay containing only primary basswood foliage, and the mid-season assay, which contained both primary and secondary tissue. In the early season array, larvae that were fed moderately damaged foliage had lower RGRs than did those fed foliage from either the low or the high damage classes. During the mid-season assay, larvae fed on primary foliage from the low and moderate damage classes had significantly lower RGRs than larvae fed on severely damaged secondary foliage. Overall, larvae from the early season assay had higher RGRs than did those from the mid-season assay.

The relative consumption rate (RCR) for each assay is listed in Table 1.

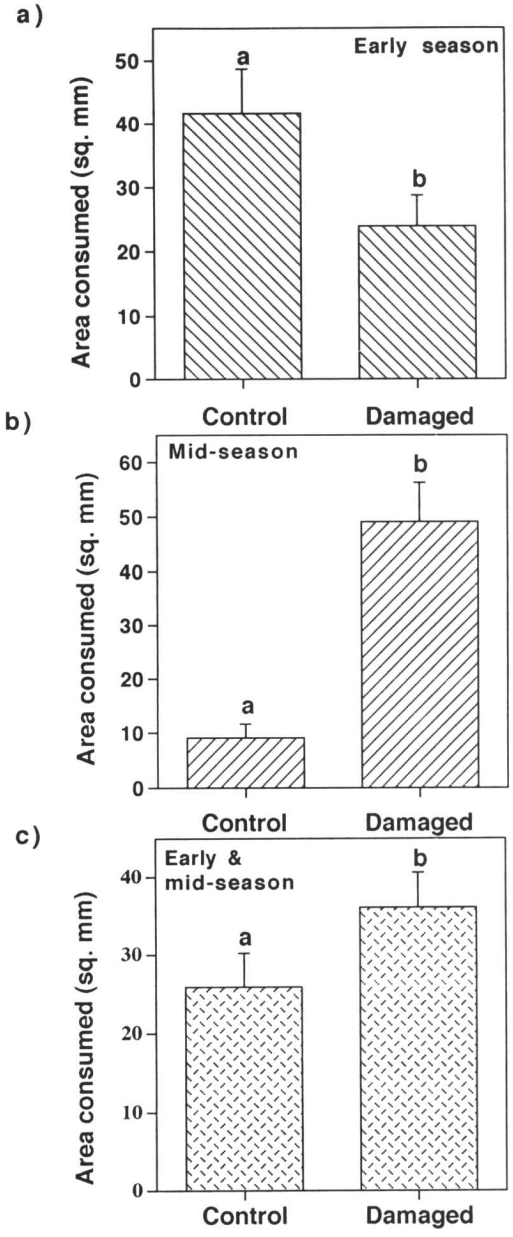

FIG. 1. Gypsy moth larval feeding preference on control (0–20% thrips-defoliated) versus thrips-damaged (>70% thrips-defoliated) American basswood foliage. Means followed by the same letter are not significantly different: (a) Early season assay: paired t test; $t = -29.384$, $df = 59$, $P = 0.0001$; (b) Mid-season assay: paired t test; $t = 28.639$, $df = 59$, $P = 0.0001$, (c) Pooled data: paired t test: $t = 41.142$, $df = 119$, $P = 0.0001$.

TABLE 1. GYPSY MOTH LARVAL PERFORMANCE ON FOLIAGE FROM TREES WITH BUD INJURY BY THRIPS

Assay date and injury level[a]	Mean RGR (mg/mg/day)[b]	Mean RCR (mg/mg/day)[c]	Stadium length (days)[d]
Early season (28 June)			
Low	0.442(0.072)a	2.063(0.247)a	5.226(0.178)a
Moderate	0.190(0.040)b	0.978(0.210)b	5.294(0.306)a
Severe	0.439(0.056)a	1.960(0.362)a	5.051(0.160)a
Mid-season (12 July)			
Low	0.168(0.027)a	0.656(0.150)a	5.000(0.258)a
Moderate	0.179(0.073)a	1.523(0.658)a	5.929(0.425)a
Severe[e]	0.342(0.050)b	1.966(0.272)b	6.127(0.250)a

[a]Low: 0–20% thrips defoliation; Moderate: 21–69% thrips defoliation; Severe: ≥70% thrips defoliation. Means (±SE) within columns within assays dates followed by the same letter are not significantly different (Fisher's protected LSD).
[b]RGR = larval biomass gained per initial larval dry weight per day. Early season assay: $F = 2.752$, $df = 2,35$, $P = 0.10$; mid-season assay: $F = 5.110$, $df = 2,35$, $P = 0.008$.
[c]RCR = leaf tissue consumed per initial larval dry weight per day. Early season assay: $F = 3.014$, $df = 2,35$, $P = 0.055$; mid-season assay: $F = 5.749$, $df = 2,35$, $P = 0.005$. Mean separations performed on log-transformed data.
[d]Time elapsed between larval molts. Early season assay: $F = 0.668$, $df = 2,35$, $P = 0.516$; mid-season assay: $F = 1.319$, $df = 2,35$, $P = 0.274$.
[e]Secondary (reflushed) tissue.

In the early season assay containing only primary foliage, consumption rates of larvae fed moderately damaged foliage were significantly lower than those fed foliage from the other damage classes, consistent with the results for the RGRs. For the mid-season assay, the RCR for larvae reared on secondary foliage from the severe damage class was significantly higher than for larvae reared on primary foliage from the low and moderate damage classes, which is also consistent with the results for the RGRs. Although the patterns of RCRs between the two assay dates were similar to the RGRs, there was no significant difference in RCR between the early and mid-season assays.

Stadium length, or the time elapsed between larval molts, was not affected by bud damage level on either assay date (Table 1). However, stadium was affected by assay date. Larvae reared in the mid-season assay remained as second instars significantly longer than did those from the early season assay ($F = 6.051$, $df = 1,71$, $P = 0.010$).

Because of the indeterminant growth characteristics of American basswood, the second collection date contained secondary reflushed foliage in the severe damage class. Table 2 lists performance parameters analyzed by tissue phenology for the mid-season assay. RGR and RCR were higher and mean stadium length longer for larvae reared on secondary rather than primary foliage.

TABLE 2. GYPSY MOTH LARVAL PERFORMANCE PARAMETERS IN MID-SEASON ASSAY ON PRIMARY VERSUS SECONDARY FOLIAGE FROM BUD-DAMAGED TREES[a]

Foliage type	Mean RGR (mg/mg/day)[b]	Mean RCR (mg/mg/day)[c]	Stadium length (days)[d]
Primary	0.156(0.023)a	0.610(0.120)a	5.073(0.222)a
Secondary	0.341(0.049)b	2.171(0.302)b	6.256(0.236)b

[a]Means (±SE) within columns followed by the same letter are not significantly different (Fisher's protected LSD).
[b]$F = 11.313$, $df = 1,71$, $P = 0.0012$.
[c]$F = 22.316$, $df = 1,71$, $P = 0.0001$.
[d]Time elapsed between larval molts. $F = 13.301$, $df = 1,71$, $P = 0.0005$.

When considering larval performance parameters for primary mid-season tissue (i.e., comparing tissue from low and moderate bud injury classes), larvae fed foliage from the low damage class had higher RGRs and RCRs than did those fed foliage from the moderate damage class (RGR: $F = 8.969$, $df = 1,35$, $P = 0.005$; RCR: $F = 3.613$, $df = 1,35$, $P = 0.065$), but there was no significant difference in stadium length.

Foliar Total Nonstructural Carbohydrate Analysis. Figure 2 illustrates foliar carbohydrate concentrations for each collection date. There was no signif-

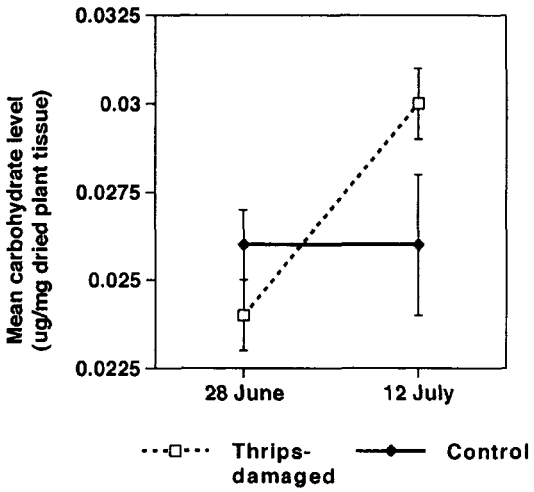

FIG. 2. Temporal changes in mean total nonstructural carbohydrate levels of introduced basswood thrips-damaged (>70% thrips-defoliated) and control (0–20% thrips-defoliated) American basswood foliage in an early versus mid-season assay.

icant difference in total nonstructural carbohydrate levels between control (0–20% thrips-damaged) and bud-damaged (>70% thrips feeding damage) tissue on the early collection date. Likewise, there was no change in carbohydrate levels in the control foliage between the two collection dates. However, on bud-damaged tissue there was an approximately 1.25× increase in carbohydrate levels between the two collection dates (least squares means, $t = 3.489$, $df = 1,35$, $P = 0.007$). There was also a 1.5× increase between bud-damaged and control foliage by the second date (least squares means, $t = 2.319$, $df = 1,35$, $P = 0.022$), with a strong date × defoliation interaction ($F = 7.132$, $df = 1,35$, $P = 0.009$).

Foliar total nonstructural carbohydrates for each damage class were affected by thrips bud-feeding in the early season and mid-season assays (Table 3). In early season primary foliage, carbohydrate levels in foliage from the moderate damage class were 0.63× less than in foliage from the other damage classes. During the mid-season assay, carbohydrate levels in secondary foliage from the severe damage class were 2.0× higher than the low damage class, and 1.2× higher than the moderate damage class, both of which were primary foliage. Secondary foliage contained significantly higher nonstructural carbohydrate levels than did primary foliage (Table 4: $F = 11.797$, $df = 1,71$, $P = 0.009$). However, within a specific tissue type, the level of bud herbivory had no significant effect on foliar carbohydrate levels.

TABLE 3. TOTAL NONSTRUCTURAL CARBOHYDRATE AND PHENOLICS LEVEL OF FOLIAGE FROM AMERICAN BASSWOOD TREES WITH BUD INJURY BY THRIPS[a]

Assay date and injury level	Carbohydates[b]	Phenolics[c]
Early season		
Low	0.008(0.001)a	5.914(0.307)a
Moderate	0.005(0.001)b	6.659(0.359)ab
Severe	0.008(0.001)a	6.930(0.212)b
Mid-season		
Low	0.003(0.0044)a	5.118(0.272)a
Moderate	0.005(0.001)ab	6.083(0.461)a
Severe[d]	0.006(0.001)c	5.431(0.163)a

[a] Means (±SE) followed by the same letter within assay dates are not significantly different (Fisher's protected LSD). Injury level as defined in Table 1.
[b] Mean total nonstructural carbohydrate levels (μg/mg dried plant tissue). Early season assay: $F = 2.644$, $df = 2,35$, $P = 0.077$; mid-season assay: $F = 3.453$, $df = 2,35$, $P = 0.035$.
[c] Mean foliar phenolics level (μmol/g dried plant tissue). Early season assay: $F = 4.055$, $df = 2,35$, $P = 0.026$; mid-season assay: $F = 3.074$, $df = 2,35$, $P = 0.610$.
[d] Secondary (reflushed) tissue.

TABLE 4. TOTAL NONSTRUCTURAL CARBOHYDRATE AND PHENOLICS LEVEL OF MID-SEASON PRIMARY VERSUS SECONDARY FOLIAGE FROM AMERICAN BASSWOOD TREES WITH BUD INJURY BY THRIPS[a]

Foliage type and injury level	Carbohydrates[b]	Phenolics[c]
Primary		
Low	0.003(0.004)a	5.660(0.638)a
Moderate	0.002(0.001)a	6.280(0.638)b
Secondary		
Severe	0.006(0.001)a	5.431(0.164)a

[a]Means followed by the same letter are not significantly different (Fisher's protected LSD). Injury level as defined in Table 1.
[b]Mean total nonstructural carbohydrate levels (μg/mg dried plant tissue).
[c]Mean foliar phenolics level (μmol/g dried plant tissue).

The relationship between larval RGR and carbohydrate levels for each assay date is illustrated in Figure 3. There was a strong positive relationship between the two factors in both the early season and mid-season assays. The relationship between RCR and carbohydrate levels was also correlated (Figure 4), but more variable than the relationship with growth rates. The inverse relationship between larval stadium length and carbohydrate levels was significant but highly variable for the early season assay ($R^2 = 0.129$, $F = 12.59$, $df = 1,35$, $P = 0.0006$) and was not significant for the mid-season assay.

Relating larval performance parameters to carbohydrate levels based on foliar phenology yielded similar results. There was a strong positive relationship in the mid-season assay between RGR and mean total nonstructural carbohydrate levels in both the primary tissue ($R^2 = 0.788$, $F = 167.45$, $df = 1,35$, $P = 0.0001$) and the secondary foliage ($R^2 = 0.869$, $F = 153.56$, $df = 1,35$, $P = 0.0001$). There was also a positive relationship between RCR and carbohydrate levels for primary tissue in the mid-season assay ($R^2 = 0.628$, $F = 74.22$, $df = 1,35$, $P = 0.0001$). The relationship between RCR and carbohydrate levels for secondary tissue was also significant but more highly variable ($R^2 = 0.354$, $F = 23.06$, $df = 1,35$, $P = 0.0001$). The inverse relationship between stadium length and carbohydrate levels was significant, but again, highly variable (primary tissue: $R^2 = 0.129$, $F = 6.676$, $df = 1,35$, $P = 0.0131$; secondary tissue: $R^2 = 0.061$, $F = 2.836$, $df = 1,35$, $P = 0.0993$).

Foliar Phenolics Analysis. Figure 5 illustrates phenolic content of foliage from each collection date. Collection date had a significant effect on foliar phenolics ($F = 9.733$, $df = 1,35$, $P = 0.0029$), and there was a strong date

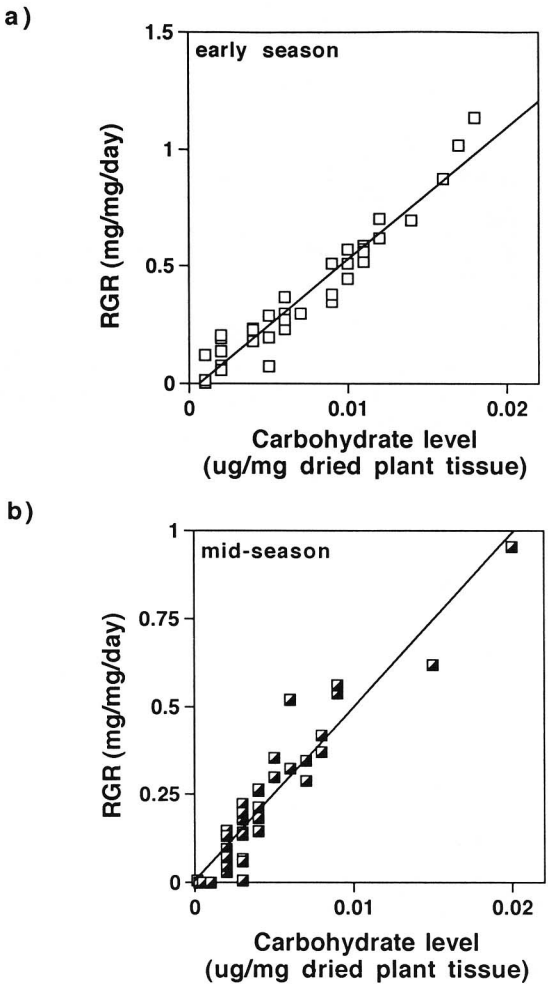

FIG. 3. Relationship between larval relative growth rate and mean carbohydrate level of thrips-damaged American basswood foliage. (a) Early season assay: $R^2 = 0.933$, $F = 96.79$, $df = 1,35$, $P = 0.0001$, $y = 56.396x - 0.035$; (b) mid-season assay: $R^2 = 0.883$, $F = 60.42$, $df = 1,35$, $P = 0.0001$, $y = 49.516x + 0.005$.

× defoliation interaction ($F = 4.912$, $df = 1,35$, $P = 0.0309$). There was a significant difference between total foliar phenolics in "control" and "thrips-damaged" tissue in early season foliage (least squares means, $t = 2.557$, $df = 1,35$, $P = 0.050$). Bud-damaged foliage had a 1.8× higher phenolics levels than did control foliage. There was also a 1.28× decrease in total phenolics in

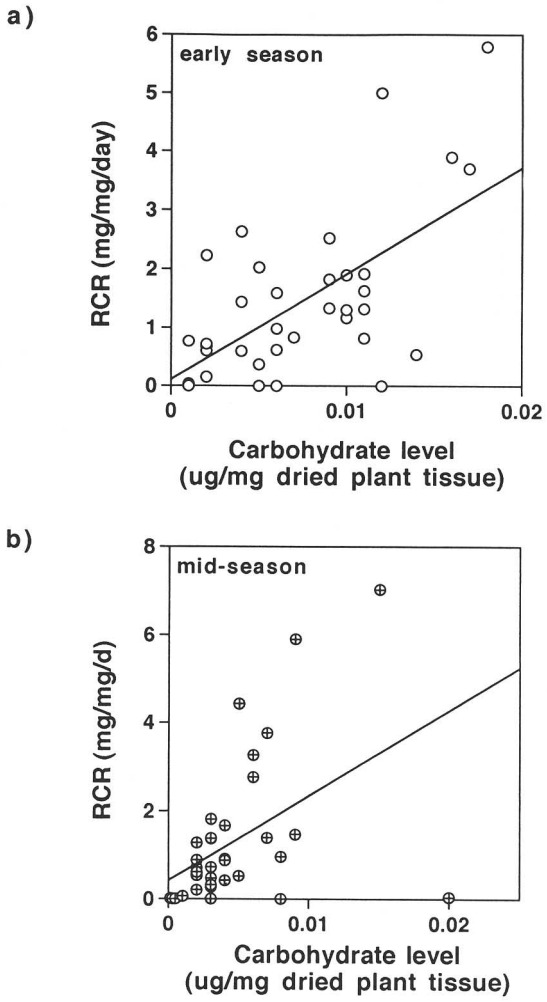

FIG. 4. Relationship between larval relative consumption rate and mean carbohydrate level of thrips-damaged American basswood foliage. (a) Early season assay: $R^2 = 0.374$, $F = 49.61$, $df = 1,35$, $P = 0.001$, $y = 181.042x + 0.105$; (b) mid-season assay: $R^2 = 0.213$, $F = 62.34$, $df = 1,35$, $P = 0.0001$, $y = 192.216x + 0.433$.

bud-damaged foliage between the two collection dates (least squares means, $t = 3.985$, $df = 1,35$, $P = 0.020$). However, there was no significant difference in phenolics levels in control tissue between the two collection dates, and no significant difference between thrips-damaged and control foliage by the second date.

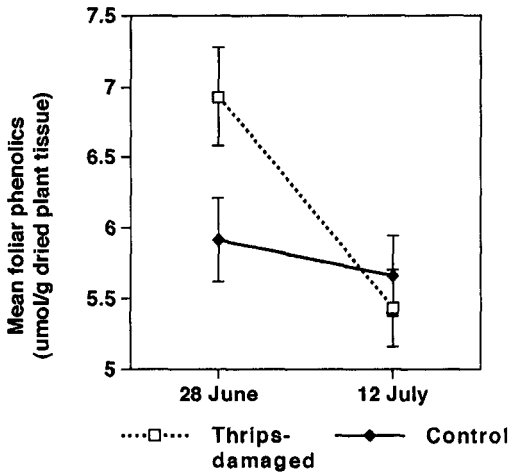

FIG. 5. Temporal changes in mean foliar phenolics levels of introduced basswood thrips-damaged (>70% thrips-defoliated) and control (0–20% thrips-defoliated) American basswood foliage in an early versus mid-season assay.

Foliar phenolic levels (Table 3) were affected by bud herbivory in the early season and mid-season assays. In the early season assay, phenolics from the severe damage class were 1.2× greater than those in foliage from the low damage class. However, in the early season assay there was no significant difference between the low and moderate damage classes and no significant difference between the moderate and severe damage classes. In the mid-season assay (Table 3), phenolics in the moderate damage class were 1.2× greater than phenolics in the low damage class, but there was no significant difference in foliar phenolics between the three damage classes.

Foliar phenology did not affect total phenolics levels. There was no difference in phenolics levels between primary and secondary foliage. However, there was a significant difference in phenolics levels based on the level of bud herbivory within the primary foliage (Table 4). Foliage from moderately damaged trees contained 1.1× higher phenolics levels than did foliage with low levels of thrips damage.

When relationships between foliar phenolics and larval performance parameters were considered separately for primary and secondary mid-season foliage, some significant but highly variable trends were observed. Increasing phenolics in primary tissue resulted in variable but significant increases in larval RGR (R^2 = 0.083, F = 4.082, df = 2,35, P = 0.049) and decreases in mean stadium length (R^2 = 0.063, F = 3.006, df = 2,35, P = 0.089). There was no relationship between phenolic content of primary foliage and RCR. Likewise, there

was no significant relationship between foliar phenolic levels in secondary tissue and any of the performance parameters.

Overall relationships between total foliar phenolics levels and larval performance parameters were highly variable and, where statistically significant, had only weak biological effects. There was no significant relationship between total foliar phenolics and any of the performance parameters in the early season assay or in the mid-season assay when both tissue types are pooled.

Foliar Fiber and Nutrient Analysis. The relationships between gypsy moth growth parameters and foliar fiber levels were highly variable (Table 5). There was no significant relationship between acid-digested fiber and larval RGR or stadium length. There was, however, a weakly significant negative relationship between foliar fiber content and gypsy moth RCR. Boron and nitrogen were the only nutrients correlated with larval growth parameters (Table 5). There was a weakly significant negative relationship between gypsy moth RGR and foliar boron levels. There was a strong inverse relationship between larval stadium length and foliar nitrogen.

DISCUSSION

Bud-feeding by the introduced basswood thrips appears to alter the phytochemistry and subsequent acceptability and suitability of American basswood foliage for larval gypsy moths. Previous effects of bud herbivory on phytochemistry have been documented (Mattson and Koller, 1983; Wagner and Blake, 1983; Redak and Cates, 1984; Clancy et al., 1988; Clancy, 1991), but few studies have examined how these changes can affect alternate feeding guilds (but see Faeth, 1986).

The reversal in larval preference between early and mid-season thrips-damaged versus control leaf discs may be attributed to several factors. Foliar total nonstructural carbohydrates, which frequently stimulate insect feeding (Schoonhoven, 1968), increased from the early to the mid-season assays. Conversely, foliar phenolics, which have been shown to inhibit gypsy moth performance (Rossiter et al., 1988), decreased from the first to the second assay date. Thus, the low carbohydrate and high phenolic concentrations caused by thrips bud feeding early in the season may have reduced larval preference. Likewise, high carbohydrate levels and low phenolics in mid-season thrips-damaged tissue may have enhanced larval preference. Changes in larval preference may also have been due to changes in nutrient accumulation or fiber content in the later foliage.

Gypsy moth larval performance was affected by thrips bud injury in both the early and mid-season assays. However, these patterns were complex and did not precisely mirror behavioral preferences. The overall higher RGRs from

TABLE 5. RELATIONSHIP BETWEEN SECOND-INSTAR GYPSY MOTH GROWTH PARAMETERS AND *T. americana* FOLIAR NUTRIENTS FROM TREES DAMAGED BY THRIPS

	Larval growth parameter[a]								
	RGR (mg/mg/day)			RCR (mg/mg/day)			Stadium length (days)		
Micronutrient	*F*	*P*	R^2	*F*	*P*	R^2	*F*	*P*	R^2
Fiber (acid digested)	1.736	0.2581	0.303	6.592	0.0621	0.622	1.119	0.3366	0.229
Al (ppm)	2.471	0.1910	0.382	0.383	0.5695	0.087	0.196	0.6808	0.047
B (ppm)	4.559	0.0996	0.533	0.569	0.4927	0.124	0.954	0.3840	0.193
Ca (% dry wt)	0.323	0.6000	0.075	1.265	0.3236	0.240	0.464	0.5329	0.104
Cu (ppm)	0.059	0.8204	0.014	0.028	0.8758	0.007	3.166	0.1498	0.442
Fe (ppm)	1.325	0.3138	0.249	4.032	0.1151	0.502	1.347	0.3103	0.252
Mg (% dry wt)	0.006	0.9380	0.002	0.110	0.7567	0.027	0.308	0.6806	0.071
Mn (ppm)	0.923	0.3910	0.188	4.980	0.8950	0.555	1.913	0.2388	0.324
N (% dry wt)	1.692	0.2632	0.297	0.0004	0.9848	0.0001	20.177	0.0109	0.835
P (% dry wt)	3.512	0.1342	0.468	2.305	0.2036	0.366	0.034	0.8620	0.009
K (% dry wt)	0.005	0.9455	0.001	0.512	0.5138	0.113	0.638	0.4692	0.138
Na(pgm)	0.153	0.7153	0.037	0.991	0.3758	0.199	4.220	0.1092	0.513
S^6 (% dry wt)	0.357	0.5826	0.082	1.045	0.3644	0.207	3.297	0.1436	0.452
Zn (ppm)	0.554	0.4980	0.122	0.560	0.4960	0.123	0.638	0.4693	0.138

[a]Terms as defined in Table 1.

the early season versus mid-season assay is consistent with other studies, which have shown that food quality tends to decline with foliar age (Scriber and Slansky, 1981). This interpretation is further strengthened by the observation that RCRs followed the same general pattern as RGRs in both assays. Secondary foliage provided a better nutritional substrate than primary foliage from the same trees later in the season. Total nonstructural carbohydrate concentrations, RCRs and RGRs were considerably greater, although larvae required more time to complete the second stadium.

Gypsy moth performance was related to total nonstructural carbohydrate levels. The patterns of thrips-induced changes in foliar carbohydrates mirror the changes in RCR and RGR. Moderately thrips-damaged, early season, primary foliage contained lower carbohydrate concentrations and produced larvae with lower RGRs than foliage from the low or high damage classes. Similarly, severely damaged, mid-season (secondary) foliage contained higher carbohydrate levels and produced faster growing larvae that consumed more tissue. Among primary plant metabolites, total nonstructural carbohydrates have been shown to be influenced by thrips feeding in other systems (Kolb et al., 1992). Foliar phenology also affected total nonstructural carbohydrate levels. Secondary tissue from the mid-season assay contained higher carbohydrate levels and resulted in greater consumption and growth. In contrast to the stimulatory effects of carbohydrates on insect success seen here, studies in other systems have shown that elevated carbohydrate levels can prolong larval development (Savopoulou-Soultani et al., 1994) and that elevated concentrations of specific carbohydrates may even cause larval mortality (Zou and Cates, 1994).

Foliar phenolics have been identified and quantified in *Tilia* species (Burden and Kemp, 1983; Roth et al., 1994). Although Roth et al. (1994) found a strong inverse relationship between phenolic glycoside concentrations in trembling aspen and gypsy moth larval performance, no such relationship was found with basswood. It has been suggested that gypsy moth larvae base foliar quality on the presence of tannins and other phenolics (Schultz, 1988), which are common components of their host plants (Barbosa and Krischik, 1987). In our study, the relationships between foliar phenolic levels and larval performance parameters were weak. However, this could vary, as the effects of compounds such as phenolics can be affected by foliar nutrient levels (Wagner and Evans, 1985) and larval growth stage (Bourchier and Nealis, 1993). Foliar phenolics in deciduous trees generally increase during the early part of the growing season and become constant after mid- to late-June (Hillis and Swain, 1959; Feeny and Bostock, 1968; Haukioja et al., 1978; Mattson and Palmer, 1988). In our study, both collection dates occurred after phenolics should have stabilized, so the observed changes were most likely due to wounding by thrips feeding.

The initial accumulation of phenolics in bud-damaged tissue may relate to induced phytohormone accumulation. Haukioja et al. (1990) and Karban and

Niiho (1995) suggest that hormonal disturbances in woody plants results in an amelioration of leaf quality, although the exact mechanisms are unknown. In our system, thrips feeding has been shown to elicit ethylene production in American basswood (Rieske and Raffa, 1995). Increased ethylene emission induces activation of phenylalanine ammonia lyase (PAL), which helps regulate phenolic synthesis following wounding (Hyodo and Yang, 1971; Yang and Pratt, 1978; Karban and Myers, 1989). The subsequent reduction in foliar phenolic concentrations suggests that early season induced defensive chemicals may soon break down as the host becomes increasingly compromised.

Figure 6 summarizes the known responses of basswood to thrips-induced bud injury and postulates some possible underlying mechanisms affecting gypsy moth response. Thrips feeding elicits a rapid threefold elevation in ethylene emission (Rieske and Raffa, 1995). This presumably contributes to the observed increases in phenolic concentrations, based on current understanding of general biosynthetic pathways (Abeles et al., 1992). Based on results with other trees (Rossiter et al., 1988; Roth et al., 1994), this could reduce gypsy moth larval feeding and development, although in our system other factors are clearly involved.

Elevated ethylene emission also exerts an indirect, time-delayed response.

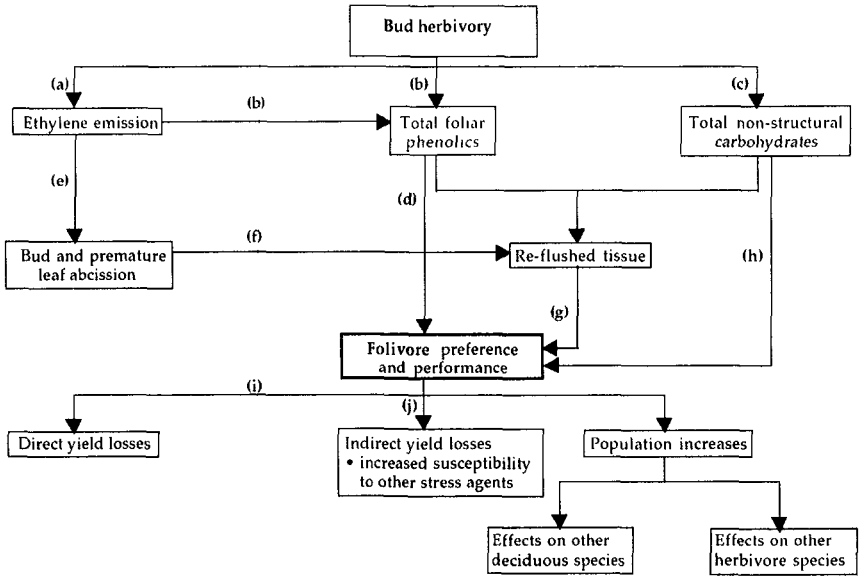

FIG. 6. Potential effects of bud herbivory on folivore preference and performance, forest productivity, and forest community structure.

Ethylene is known to induce bud and leaf abscission (Berrie, 1984), which promotes reflushing and the production of secondary foliage, which in turn favors gypsy moth feeding and development. A second delayed response is manifested in the form of increased total nonstructural carbohydrates. Our results show that elevated carbohydrates facilitate gypsy moth larval feeding and development.

Interactions between feeding guilds, such as the one described here, could potentially have strong impacts on insect population dynamics, host-plant performance, and forest community structure. In this system, thrips feeding could augment the direct injury exerted by a subsequent herbivore on host plants or increase plant susceptibility to other stresses (Kuhlman, 1971; Dunbar and Stephens, 1975; Campbell and Sloan, 1977). Conversely, plant response to bud injury might reduce the folivores' success during the critical early instars. This could be particularly important in the case of gypsy moth, which frequently abandons unpalatable plants during the first stadium. Either increased population densities resulting from more successful performance or increased emigration due to unsuitable foliage by a generalist folivore such as the gypsy moth could cause indirect effects among plant species in mixed deciduous forests.

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A VERSATILE METHOD FOR ON-LINE ANALYSIS OF VOLATILE COMPOUNDS FROM LIVING SAMPLES

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Abstract—A system especially designed for the on-line analysis of volatiles emitted by living aerobic organisms is described. The instrument consists of a modified thermal desorber and utilizes the purge and cold trap (PCT) technique coupled with gas chromatography–mass spectrometry. The system enables the use of a variety of sample vessels especially adapted to individual test organisms. Quantitative analyses of some volatile semiochemicals revealed that when only nanogram amounts of these compounds are evaporated at room temperature for a period of 10 min, they may be trapped and detected by this device. Emission of volatiles from two insect species and a plant species have been studied in detail by using this system.

Key Words—Purge and cold trap technique, gas chromatography–mass spectrometry, volatile analysis, *Chrysomela vigintipunctata, Nasutitermes nigriceps, Ulmus carpinifolia*.

INTRODUCTION

Dynamic headspace analysis is a convenient method for the enrichment of volatiles from complex matrices, combining the advantage of low detection limits with minimal sample preparation (Vieths, 1992). The method, which is also called the purge-and-trap technique, is often used for analysis of insect and plant volatiles (Golub and Weatherston, 1984). The different applications of this technique have in common that a gas stream continously flows through the vapor space (headspace) of a sample and purges volatiles emitted by the sample to a trap. The trap may be either an adsorption trap or a cold trap. Immobilized

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volatiles are usually removed from the trap by solvent elution or thermal desorption and subsequently analyzed by gas chromatography (GC) (Drozd and Novak, 1979; Leahy and Reineccius, 1983; Vieths, 1992). The use of solvents for removal of trapped compounds has some disadvantages in comparison to thermal desorption: (1) Dilution of analytes causes loss of efficiency. (2) Highly volatile compounds may be obscured by the solvent peak. (3) Solvents may contaminate the sample (Núñez and Gonzáles, 1984). On the other hand, solvent elution makes volatiles available not only for chemical analysis, but also for bioassays.

On-line analysis of volatiles using cold traps has, up to the present, only been described for dead organic matrices. To keep the organisms alive, air has to be used as a purge gas. However, oxygen may damage the GC column and mass spectrometric detectors. Thus, in an on-line arrangement remaining air has to be removed from the collecting system prior to GC analysis. This paper describes a purge-and-trap arrangement with cryotrapping and successive thermal desorption, especially modified for on-line analysis of volatiles from living aerobic systems by GC-mass spectrometry (GC-MS).

METHODS AND MATERIALS

Instrumental Setup

The arrangement is based on a computer-controlled Tekmar AEROTrap 6000 thermal desorber (CE Instruments, Mainz-Kastel, Germany) equipped with a liquid nitrogen cooled cryotrap. The instrument was designed originally for thermal desorption of adsorption tubes filled with porous polymers such as Tenax TA, but was modified by us for on-line sampling of volatiles from living samples. It consists of the following key elements (Figure 1a): (1) Purge Gas Supply—the purge gas (synthetic air, FID-quality, additionally purified by a charcoal trap) is led via stainless steel tubing equipped with Cajon Ultra-Torr fittings (Best, München, Germany) which can be connected to any 6 mm OD tubes without use of a tool. (2) Sample flask—the use of hand screws allows the attachment of sample vessels appropriate to the test organisms. In most cases, we use 500-ml wash bottles (Figure 2). For small samples, it is optimal to use only short pieces of 6-mm-OD glass tube for sample storage during the sampling process because the danger of contamination increases with the size of the sample vessels. (3) Three-port valve—the additional valve is positioned outside the AEROTrap housing but is controlled by its software. The switching of this valve enables the system to be purged with helium in order to remove oxygen residues from the purge process prior to GC analysis. (4) Sample transfer line—The sample gas is led through this heatable transfer line (1/16-in. nickel tubing) into the AEROTrap instrument. (5) Six-port valve—this heatable valve controls the gas flows inside the instrument. (6) Moisture control system (MCS)—

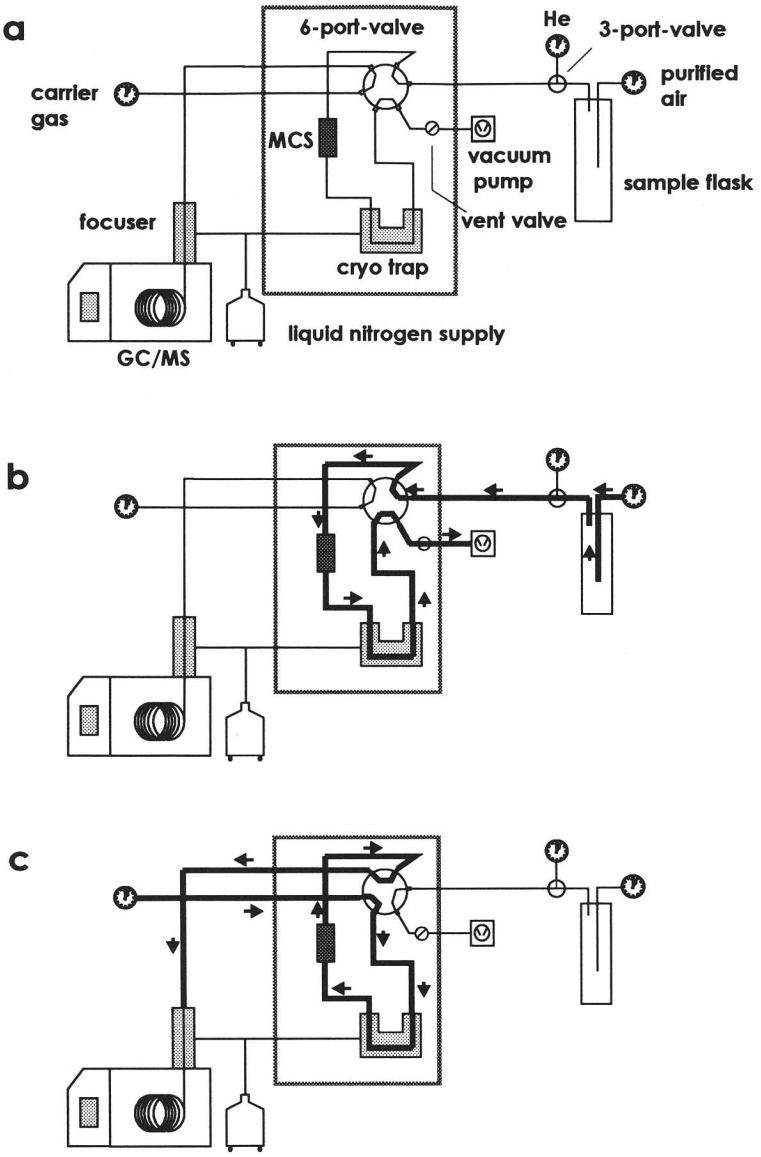

FIG. 1. Schematic diagram of the PCT/GC-MS arrangement (a) and gas flows during sample desorb (b) and trap desorb (c) steps.

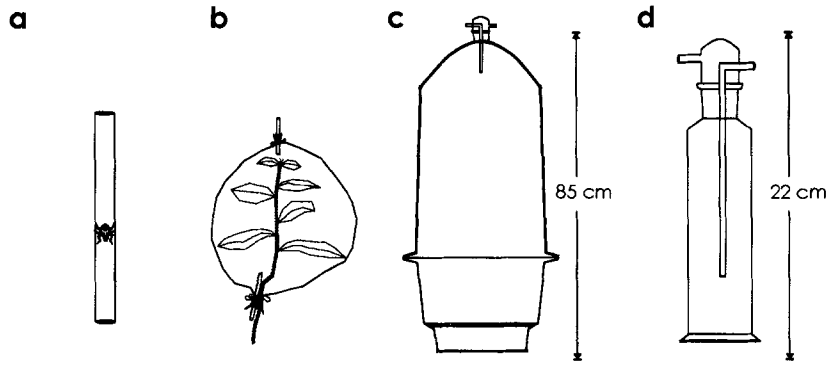

FIG. 2. Possible vessels for sample storage during headspace sampling: (a) glass tube, 6 mm OD, for single small specimens; (b) PET-film tube with 6-mm glass tube adapters for intact plants; (c) glass vessel (ca. 56 liters) consisting of a plane flanged desiccator base with a bell-shaped lid, equipped with a NS 60/46 wash bottle insert; and (d) 500-ml wash bottle.

This is a modular ambient condensate system that removes moisture from the gas stream going to the GC. (7) Cryotrap—the cryotrap consists of a 6-in. × 1/8-in. stainless steel tube filled with glass beads. Tubes filled with Tenax or charcoal are also available. It is cooled by liquid nitrogen. The working temperature (applied range from −50 to −150°C) depends on the volatility of the analytes and is controlled by magnetic valves. (8) Carrier gas supply—the AEROTrap instrument has a separate carrier gas connection. In the "trap desorb" mode of the six-port valve, the condensed analytes are transferred from the cold trap to the GC by the carrier gas via another heatable transfer line (0.32-mm uncoated fused silica tubing). (9) Focuser—the GC is equipped with a cryofocusing module that is cooled by liquid nitrogen. This module is kept cool during the internal trap desorption to focus the analytes to produce narrow starting bands. (1) Vent valve—this valve is controlled by a manual switch to interrupt the purge gas flow during volatile equilibration. (11) Vacuum pump—a model MZ2 vacuum pump (Vaccubrand, Wertheim, Germany) is installed at the outlet of the AEROTrap to support the gasflow which streams through the narrow tubing (1/16-in. nickel) inside the instrument. By this means, purge gas flows up to 250 ml/min are possible without increasing pressure inside the sample flask. (12) Liquid Nitrogen Supply—the liquid nitrogen is stored in an Apollo 50 Dewar vessel (Messer Griesheim, Krefeld, Germany), and the cooling temperatures of the trap and the focuser are controlled by the software via magnetic

valves. The sampling time is limited by the capacity of the Dewar vessel to 8–10 hr.

Operating Steps

The operating sequence of the instrument consists of the following main steps between which the gas flows differ due to different valve configurations: (1) Standby—the sample gas is led through the instrument without trapping, and the internal trap is not cooled. For volatile equilibration, the purge gas flow is interrupted by the vent valve. (2) Trap cooldown—the internal trap is cooled down to its operation temperature. (3) Sample desorb (Figure 1b)—the sample gas is led through the instrument, passes through the six-port valve and enters the internal trap where the volatile constituents are condensed. The sample desorption time varies between 1 min and several hours and depends on the content of volatiles in the samples. (4) MCS cooldown—before the internal trap is heated, the MCS is cooled to its operation temperature (50°C) in order to remove any trapped water prior to GC analysis. At the same time, the three-port valve switches and the whole system is purged with helium, and the remaining oxygen from the sampling process is removed. (5) Cryomodule cooldown—during this step, the cryomodule is cooled to its low temperature setpoint, which normally correlates with the internal trap temperature. (6) Trap desorb—prior to trap desorption, the six-port valve rotates and the gas flow changes as shown in Figure 1c. The internal trap is flash-heated to its upper setpoint (250°C), and the carrier gas transfers the desorbed analytes to the cryomodule where they are condensed again. Most of the condensed sample water is removed by the MCS. (7) Cryo inject—during this step the cryomodule is flash-heated within 1 min to its upper setpoint (250°C) in order to release the immobilized volatiles for GC-MS analysis. (8) Bake—finally all heatable parts of the instrument are heated to a set temperature in order to remove any residues.

GC-MS Analysis

Analytical separations were performed on a Fisons model 8060 GC, and mass spectra were obtained on a Fisons MD800 quadrupole mass spectrometer (CE Instruments). Analyses were carried out by using a 30-m × 0.32-mm-ID. DB-5ms fused silica column, film thickness 1.0 μm or 0.25 μm (J&W/Fisher Scientific, Wiesbaden, Germany) with helium as carrier gas (forepressure 10 kPa). The temperature program was started at 40°C, held for 1 min, and raised at 4°C/min to 280°C. The column effluent was ionized by electron impact ionization (EI) at 70 eV, and eluted compounds were identified by comparing obtained spectra with NIST library spectra.

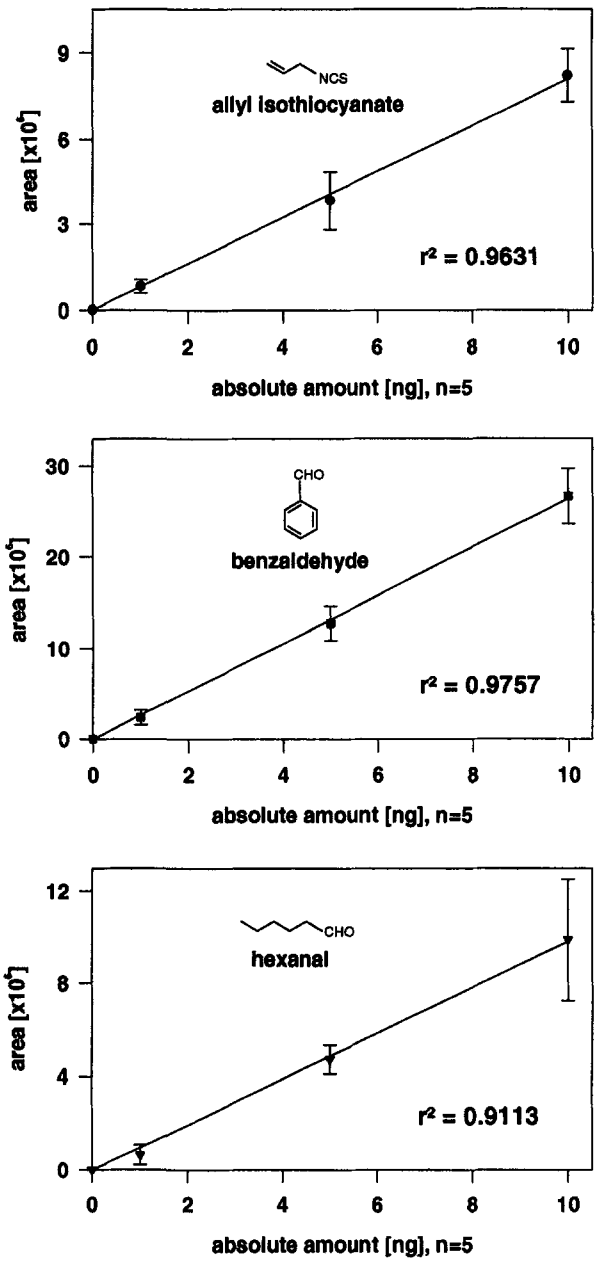

FIG. 3. Calibration curves of three volatile semiochemicals in water. Peak areas obtained by PCT/GC-MS analysis of three different standard solutions of each compound (N = 5).

RESULTS

Quantitative Analyses

To demonstrate that the instrument is suitable for the analysis of nanogram amounts of volatile semiochemicals, we prepared standard solutions containing hexanal, benzaldehyde, and allyl isothiocyanate at concentrations of 100, 500, and 1000 ng/ml in water. Using a 10-cm glass tube, 6 mm OD (Figure 2a), as a sample container, we injected 10 μl of each standard solution ($N = 5$), representing 1, 5, and 10 ng. The purge gas flow was adjusted to 40 ml/min for 10 min, and the trapping temperature was $-150°C$. The resulting calibration curves are shown in Figure 3.

To elucidate recovery ratios, we prepared standard solutions containing the three compounds at concentrations of 1, 5, and 10 ng/μl in dichloromethane. Aliquots (1 μl, splitless, $N = 3$) of these solutions were analyzed under the same conditions using a conventional split/splitless injector. Recovery ratios were estimated by comparison of the resulting peak areas with those obtained by the purge and trap procedure. Recovery ratios are summarized in Table 1.

Applications

Figure 4a–c shows three typical total ion current chromatograms obtained with the PCT/GC-MS technique (for the operation conditions see Table 2). Larvae of the leaf beetle *Chrysomela vigintipunctata* emit salicylaldehyde as a major defensive component of their exocrine glandular secretions (Pasteels et al., 1982). This compound can be identified from a single specimen within a purge time of 1 min (Figure 4a). Soldiers of the termite *Nasutitermes nigriceps* secrete several terpenes as defense compounds from their frontal glands (Valterova et al., 1987). The analysis of the headspace volatiles from 40 specimens revealed that, in addition to the described terpenes, some aromatic compounds are also present (Figure 4b). Several of the so-called general green leaf volatiles were detected in the headspace of a single elm twig (10 leaves, weight about 2.0 g, see Figure 4c).

TABLE 1. RECOVERY RATIOS OF PURGE-AND-TRAP PROCEDURE FOR NANOGRAM AMOUNTS OF THREE SEMIOCHEMICALS

Compound	Ratio (%)		
	1 ng	5 ng	10 ng
Hexanal	38 ± 20	52 ± 6	53 ± 12
Allyl isothiocyanate	28 ± 8	34 ± 8	34 ± 3
Benzaldehyde	49 ± 17	64 ± 9	63 ± 7

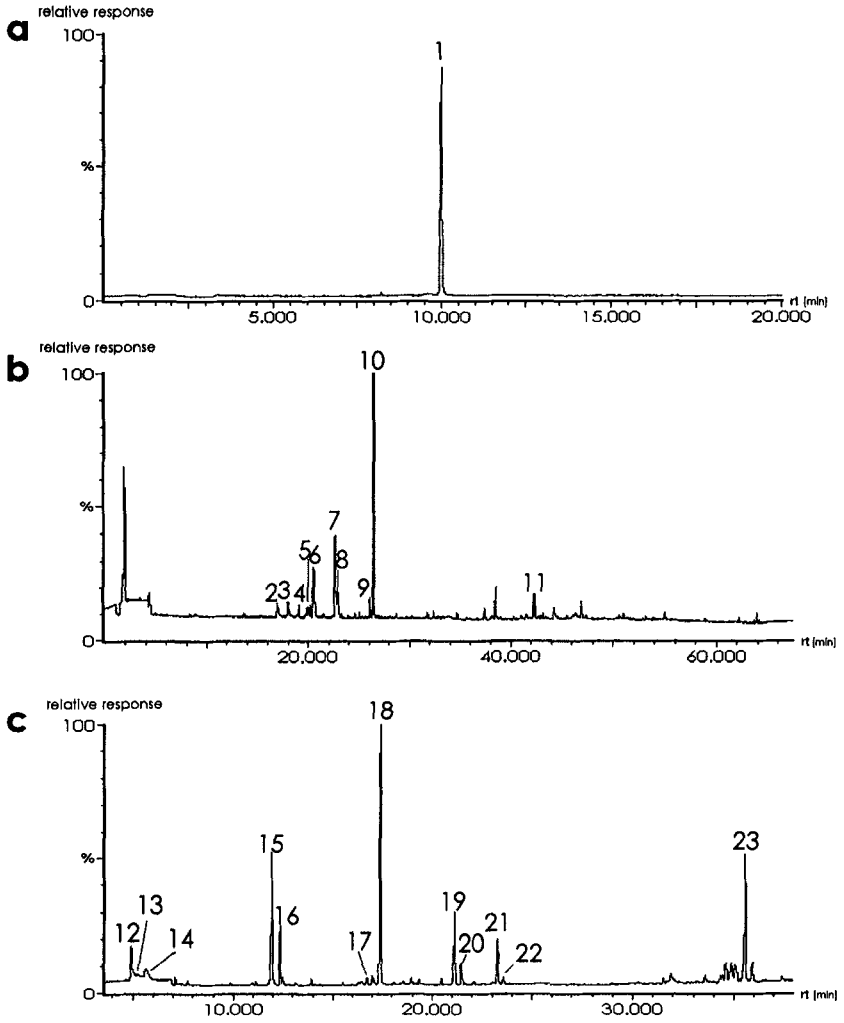

FIG. 4. (a) Normalized total ion current chromatogram (TIC) of volatiles emitted by a single larva of *Chrysomela vigintipunctata*; 1 = salicylaldehyde. (b) TIC of volatiles emitted by 40 soldiers of *Nasutitermes nigriceps*; 2 = α-pinene, 3 = camphene, 4 = benzaldehyde, 5 = α-methylstyrene, 6 = myrcene, 7 = *p*-cymene, 8 = limonene, 9 = 4-methylbenzaldehyde, 10 = *p*-cymenene (contamination), 11 = unknown sesquiterpene. (c) TIC of volatiles emitted by an elm twig; 12 = 2-hexenal, 13 = 3-hexen-1-ol, 14 = 1-hexanol, 15 = 3-hexen-1-yl acetate, 16 = hexyl acetate, 17 = 3-hexen-1-yl propanoate, 18 = unknown terpenoid, 19 = 3-hexen-1-yl butanoate, 20 = hexyl butanoate, 21 = 3-hexen-1-yl 2-methylbutanoate, 22 = hexyl 2-methylbutanoate, 23 = α-farnesene.

TABLE 2. SAMPLES AND OPERATING CONDITIONS

Sample	Amount/number	Purge gas flow (ml/min)	Trapping temperature (°C)	Purge time (min)
Chrysomela vigintipunctata	1 larva	50	−100	1
Nasutitermes nigriceps	40 soldiers	50	−100	30
Elm (*Ulmus carpinifolia*)	1 twig with ca. 10 leaves	150	−100	30

DISCUSSION

For chemoecological studies, many different systems have been described for enrichment and analysis of volatiles (Boland et al., 1984; Golub and Weatherston, 1984). These devices often have to be adapted to the organisms being investigated. The PCT instrument described here provides great flexibility. The system may be arranged to suit the organisms investigated since sample vessels may be changed easily according to the demands of the organism. By this means, it is possible to keep test organisms under near natural conditions and to analyze nanogram amounts of volatiles emitted by them. The short analysis time and the low detection limits may even allow correlation of observed behaviors (e.g., defense actions, premating behavior) with changes in the composition of the vapor phase.

The sampling parameters must be optimized for each organism. Abiotic parameters that may influence the results are, e.g, flow rate and total volume of the purge gas, cold trap temperature, sampling time, and the packing material of the trapping tube. Suggestions for their optimization have been provided by several authors (Núñez and Gonzáles 1984; Badings et al., 1985; Etievant et al., 1986; Jursik et al., 1991).

One problem that might occur is blockage of the trapping tube by moisture freezing during analysis of aqueous samples under long sampling times. Additionally, contaminated blank runs are obtained when large vessels are used for sample storage. This is due to the high amount of laboratory air in the sample gas, which cannot be removed quantitatively without an unacceptable time requirement. Recently, Bestmann et al. (1996) compared different techniques for volatile analysis pointing out the favorable properties of the PCT technique for analysis of highly volatile compounds.

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A VERSATILE AND QUANTITATIVE VOLATILE-DELIVERY SYSTEM FOR LABORATORY BIOASSAYS

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Abstract—A versatile moving-air system is described for delivering volatiles into a wind tunnel or other bioassay device. The system controls up to four volatile sources at one time. There is a calibrated, adjustable splitter for each source so that any percentage of a source's airstream, or none of it, can be directed to the system outlet at any moment. Thus, the system allows the sample volatiles to be bioassayed in any order and at any level or in mixtures of any desired proportions. Volatile sources of many types can be used, including single chemicals in slow-release formulations, mixtures of chemicals, or volatiles from living organisms. The volatile stream can be sampled by solid-phase microextraction (SPME) just before it enters the wind tunnel. Analysis of the SPME sample by gas chromatography allows absolute delivery rates of volatile components to be calculated. System performance was characterized with physical measurements and with bioassay experiments involving *Carpophilus humeralis* (F.) (Coleoptera: Nitidulidae). One bioassay experiment demonstrated how volatiles from a microbial culture (fermenting bread dough) and a synthetic counterpart (an aqueous solution of acetaldehyde, ethanol, 1-propanol, isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and ethyl acetate) could be compared at a range of dose levels, with just one sample of each type. These natural and synthetic volatile sources delivered very similar amounts of the above compounds and produced nearly identical dose–response curves. In another experiment, three bread dough volatiles (ethanol, acetaldehyde, and ethyl acetate) were tested in mixtures. Each component was used at four different levels (giving a total of 64 experimental treatments), but just one physical sample was needed for each chemical. The experiment provided clear information about response thresholds and inter-

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actions among these host volatiles. The volatile delivery system is versatile, easy to operate, and can be constructed from inexpensive materials.

Key Words—Volatile compounds, bioassay, manometer, Nitidulidae, *Carpophilus humeralis*, solid-phase microextraction, gas chromatography, wind tunnel.

INTRODUCTION

Insects experience highly complex blends of volatile chemicals from a multitude of sources as they locate feeding sites and mates under field conditions, and understanding how insects utilize chemical information has been a challenge of great basic and practical importance. Pheromones have been the most intensively studied of these volatiles. One aspect of this research has been to develop reliable dispensers, such as rubber septa, that can deliver pheromones at known and fairly uniform rates for field or laboratory studies (Heath et al., 1986; McDonough, 1991). The ability to control and measure pheromone delivery has contributed significantly to the current understanding of pheromone biology.

Host-related volatiles are now receiving increased attention. These blends of chemicals often involve a far greater range of volatilities, polarities, and concentrations than is typical for pheromones. As with pheromones, being able to control and measure delivery of host volatiles during experiments will be important to understanding how they function, but the techniques developed for pheromones (such as using rubber septa) are not often appropriate for studies of host volatiles. Techniques for handling host volatiles per se have not progressed as far as those for pheromones.

In studies of fruit fly attractants (e.g., Robacker and Flath, 1995, and references therein), test solutions were applied to pieces of filter paper for bioassays. Great care was taken to ensure that the solutions of synthetic volatiles and their natural counterparts had comparable concentrations, but the absolute rates at which the compounds reached the test insects were not measurable. Lin and Phelan (1991) allowed their samples to diffuse from bottle openings into their wind tunnel. They were able to measure static headspace concentrations in the bottles and to closely match the natural samples with synthetic blends. However, the absolute rates at which the compounds entered the bioassays could not be known.

One technique for delivering known and uniform rates of highly volatile compounds is by evaporation from glass capillaries of carefully chosen lengths and diameters (Whitman and Eller, 1992), an extension of a concept developed earlier for pheromones (see Swenson and Weatherston, 1989). This technique made it possible to study dose responses, an experimental dimension that is crucial to understanding the effects of components. Still, the calibration proce-

dure was cumbersome, and a number of dispenser configurations were required to cover the range of compound volatilities encountered in the study.

In this paper, a volatile delivery system is described that allows the kinds of experiments noted above to be conducted with advantages in speed and flexibility of use. Furthermore, it allows absolute delivery rates of the chemicals to be measured. Sample volatiles are carried by moving airstreams, and up to four samples can be controlled at one time. There is a calibrated, adjustable splitter for each source so that the sample airstreams can be bioassayed at any levels or in any combinations. The volatile sources may involve single components or mixtures of any complexity and may be either natural or synthetic.

Solid-phase microextraction (SPME) is used to analyze the airstream so that delivery rates of volatiles may be calculated. SPME is a relatively new technique for sampling organic compounds from gas-phase or aqueous systems (reviewed by Pawliszyn, 1997). Robacker and Flath (1995) demonstrated the value of SPME to chemical ecology by analyzing fruit fly attractants in static headspace samples. Malosse et al. (1995) used SPME to observe pheromone release from live weevils over time; their study was novel in that a moving airstream was sampled, although absolute quantitation was not done. Bartelt (1997) showed how static headspace concentrations of typical host volatiles could be determined by SPME. Martos and Pawliszyn (1997) demonstrated that SPME measurement of analyte concentration in moving air is essentially the same as in static air. Quantitation of airstream components can be accomplished at nearly the same time that bioassays are conducted, and it is now possible to relate behavioral observations to absolute levels of volatile blend components.

The volatile-delivery system is especially useful for conducting dose–response studies with natural or synthetic materials, for determining whether a synthetic mixture of chemicals "accounts" for the bioassay activity of a natural attractant, and for studying the effects of individual components in volatile blends. The system was developed with host volatiles in mind, but it is also appropriate for studies with pheromones. Construction, operation, and calibration of the system are presented below. Bioassay experiments with the apparatus are also described to illustrate some of its practical features and possibilities. The nitidulid beetle *Carpophilus humeralis* (F.) was used for the bioassay experiments.

METHODS AND MATERIALS

Description of Volatile-Delivery System

Air Source and Samples. Air from a compressed air tank and regulator is first passed through a cartridge of adsorbant such as Super-Q (Supelco, Belle-

fonte, Pennsylvania) or activated charcoal to remove any organic contaminants and is then passed to a bank of five needle valves. Our valves have attached flowmeters, and this is convenient for monitoring flows but is not necessary. Four of the valves are permanently set to 9 ml/min and deliver air to the sample containers, and the fifth is set to 80 ml/min as a source of makeup air. Airflow rates were measured with a digital flowmeter (Digital Flow Check, Alltech, Deerfield, Illinois). These five airstreams enter the volatile delivery system, part of which is shown diagrammatically in Figure 1. The sample source in the figure is an aqueous solution of a volatile chemical. Other types of samples are also possible, as described below.

Splitter. The airstream from the sample source enters an adjustable splitter (Figure 1), which allows any desired percentage of the sample (0–100%) to eventually reach the bioassay arena. The key component of the splitter is an airflow restrictor (a fine syringe needle). The restrictor needle creates a small but measurable back pressure in the sample air line; this pressure is read on an open-end manometer. The amount of sample actually flowing through the needle is nearly proportional to the amount of back pressure, and this is controlled with a valve (V1 in Figure 1). When the control valve is opened slightly, the back pressure is partially relieved, the amount of sample flowing through the restrictor needle is reduced, and the rest of the sample stream is diverted through the valve into the waste line. The waste line is maintained under a slight vacuum, about −2 mm water, as described below. Once the splitter is calibrated, any desired flow rate (expressed as a percentage of incoming sample flow) can be obtained by adjusting the control valve to give the appropriate manometer reading. Three valve settings, flow rates, and manometer readings are shown as examples in Figure 2.

Mixing Zone. Only one splitter is shown completely in Figure 1, but each of the four sample airstreams has its own splitter. The restrictor needles of all four splitters pass through a single Teflon-lined GC septum and end in a silanized glass tee (the mixing zone), where the effluent from the four splitters is combined (Figure 1).

Also passing through the septum is a fifth needle carrying the stream of clean makeup air (80 ml/min). With no makeup air and very low splitter flow rates (e.g., 0.1 ml/min), it would take a very long time for a sample to fill the tubing and finally emerge into the wind tunnel. The flow of makeup air ensures that any sample entering the mixing zone is rapidly swept all the way to the wind tunnel.

On/Off Valve. A second valve (V2 in Figure 1) connected to the mixing zone tee controls the destination of the effluent. When the valve is off (closed), the mixture of volatiles and makeup air moves into the wind tunnel, but when it is on (open), the mixture is diverted to the waste line. Because the waste line has a slight vacuum, any sample present in the wind-tunnel line is drawn back

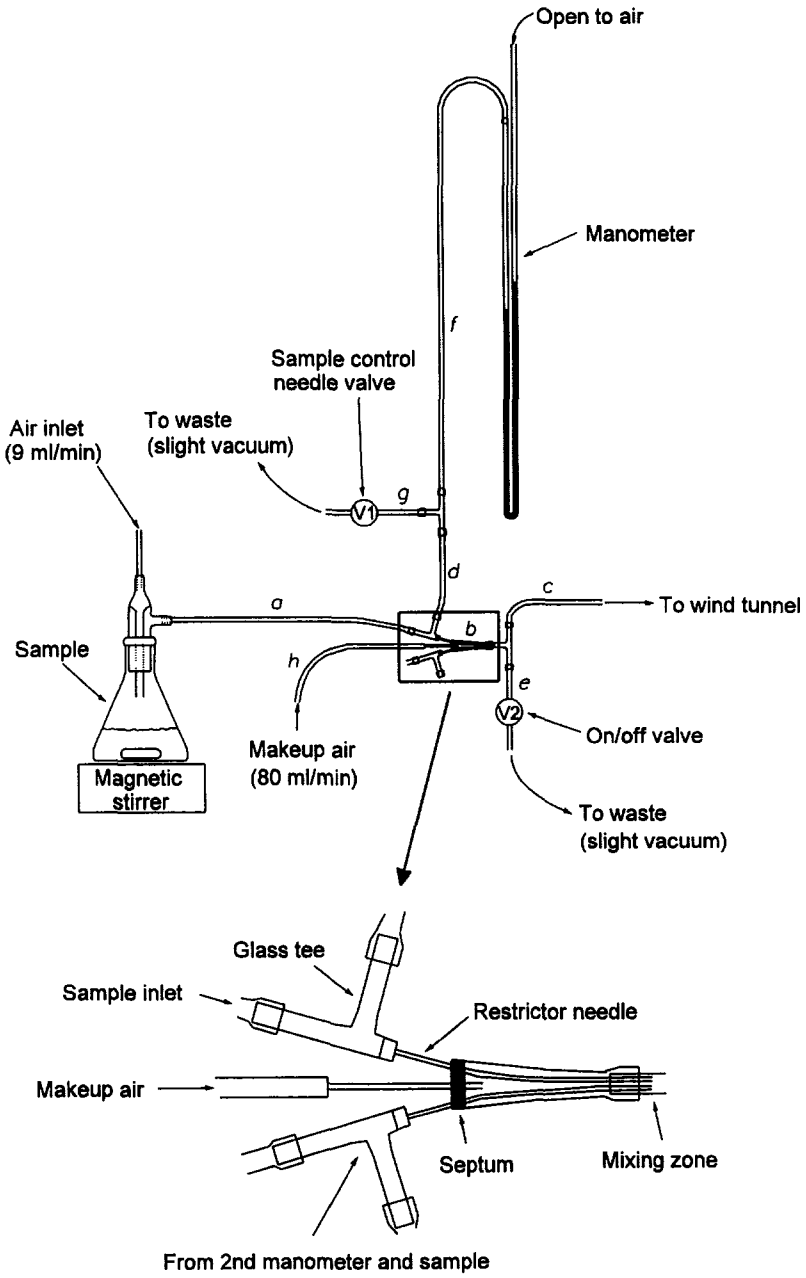

FIG. 1. Diagrammatic view of volatile-delivery system. Only one of the four adjustable splitters is shown completely. Tubing sections marked with letters are described in the text.

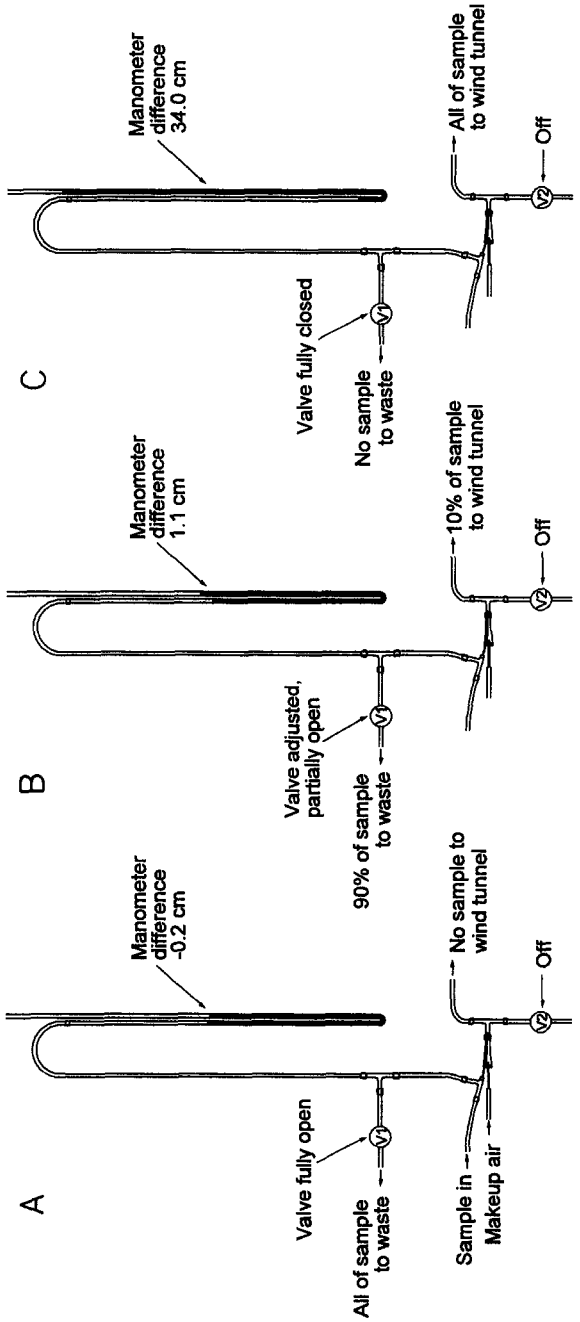

FIG. 2. Three possible settings for splitter control valve (V1) and resulting sample flows and manometer readings. (A) With valve fully open, no sample goes to wind tunnel and all flow is drawn off to waste. (B) Valve is partially open; at this setting, 10% of the sample enters wind tunnel. (C) Valve is fully closed, and all of sample stream enters wind tunnel. In all three examples, 100% of the makeup air passes into the wind tunnel.

and purged when the valve is open. This valve allows flow to the wind tunnel to be turned off between tests and during splitter adjustments.

Prototype System. An earlier, prototype splitter system had only two sample/manometer units, and the restrictors had a larger internal diameter. For this system, flow rate through the sample container had to be higher (80 ml/min), and at maximum flow through the restrictor, the manometer deflection was only 20 cm. The concept of operation was the same in both cases and data are included here from both systems, but only the later system is described in detail.

Volatile Delivery System Materials

Sample Containers. Typical sample containers are flasks having inlet/outlet adapters and ground-glass joints (24/40) (see Figure 1). Flask sizes between 50 and 1000 ml have been used, depending on the particular application, but the smaller sizes allowed splitter pressures to stabilize more quickly. When the air volume in a sample flask was 50 ml or less, the pressure and splitter flow stabilized within 1 min of the valve adjustment. Alternatively, formulated samples (e.g., rubber septa) were held in a linear tube. This was 10 × 1.5 cm in size, had 0.5-cm inlet and outlet fittings, and had a 14/35 ground glass joint through which a septum could be introduced.

Materials and Connections. All tubing directly between the volatile source and the wind tunnel was of Teflon (Cole Parmer, Vernon Hills, Illinois). Paths of volatiles were kept as short as possible. The sample inlet tube (*a* in Figure 1) was 2-mm-ID, 20-cm-long Teflon tubing. The 3-cm section of Teflon tubing between the septum and glass tee mixing zone (*b* in Figure 1) was 6 mm ID and was flared to 8 mm on one end to receive the septum. (A pin passing through the edge of the septum secured it to the tubing). The outlet to the wind tunnel (*c* in Figure 1) consisted of 30 cm of 4-mm-ID Teflon tubing connected to a 120-cm section of 3-mm ID Teflon tubing.

Connections between Teflon tubing and glass or between different sizes of Teflon tubing were snug, and joined pieces were always telescoped together. A piece of Tygon tubing of appropriate inside diameter was fitted over each joint to ensure that it was airtight. Leaks could be detected with the electronic flowmeter; no leak was present if total flow into the system equaled that entering the wind tunnel when all valves were closed.

Side branches of the system leading to valves or manometers (Figure 1) had Teflon tubing immediately adjacent to the bioassay sample stream, but more distal portions were prepared from Tygon, which is more flexible and easier to manipulate, if less inert. Sections *d* and *e* in Figure 1 were of 2-mm-ID, 20-cm-long Teflon tubing, while sections *f*, *g*, and *h* were Tygon tubing of 3 mm ID and lengths of 30, 2, and 50 cm, respectively. All sections of the system made from Tygon tubing either could not contact sample volatiles (*h*), were

dead air zones (*f*), or carried airflow toward the waste vacuum (*g*); the volatiles passing to the wind tunnel contacted only Teflon tubing surfaces.

Glass tees were 4 mm ID; arms were trimmed to the shortest practical lengths, and tees were silanized for 30 min at 40°C with 5% dichlorodimethylsilane in toluene. The airflow restrictors were stainless steel needles, 5 cm × 0.5 mm OD with a bevel tip (SGE brand, Alltech #85274, Deerfield, Illinois). The metal hub of the needle was pushed into a 5-mm length of Teflon tubing, and the covered hub then fitted snuggly inside the glass tee.

Each manometer consisted of two 50-cm pieces of straight glass tubing of 2 mm ID. The U-shaped curve at the bottom was made from Tygon tubing. The manometers were filled to a height of about 25 cm with water. They also served as pressure-relief valves for the system; the water would be expelled through the open end if a blockage ever occurred in the airstream. A centimeter ruler was mounted behind each manometer. A dye could be added to the water to improve visibility.

Wind-Tunnel Outlet. The wind tunnel was as described previously (Bartelt et al., 1990). The Teflon tubing from the splitter entered into the wind tunnel vertically, through a small hole in the floor, and was terminated with an L-shaped piece of 2-mm-ID silanized glass tubing. This outlet was pointed downwind, and a piece of fine brass screening was fitted over the opening to prevent beetle entry. The outlet was located along the center line of the wind tunnel, 40 cm above the floor, and 20 cm from the upwind end. A circular piece of tan cardboard, 8 cm in diameter, served as a landing target for bioassay beetles. The outlet for volatiles passed through a hole at its center, and the plane of the cardboard was perpendicular to the direction of wind flow.

Mounting of Components. The manometers, valves, and glass components were mounted securely on a wooden frame so that air-tight connections would be held immobile, valves could be adjusted conveniently, and manometer readings could be made easily. Unlike the diagrammatic representation in Figure 1, the four splitter needles and attached glass tees were mounted in a horizontal plane, so that the sample sources could be set on a table and the associated tubing would converge at the septumlike spokes of a wheel. The side arms of the tees, leading to the control valves and manometers, were all vertical. Generally, the locations of the components were not critical to operation.

Vacuum Source. The valve for the house vacuum was opened, allowing air to enter at a rate of about 1.5 liter/sec. The vacuum line was attached to a container with two other ports. One of these was connected through a manifold to all of the waste lines from the splitter system with Tygon tubing. The other, having an inside diameter of 0.5 cm, was left open to the air. The pressure at the manifold, relative to the outside, was about −2 mm water. This vacuum was slight, but there was enough capacity to accept the entire flow from the

splitter system when the on/off valve (V2) was open, preventing any volatiles from entering the wind tunnel.

Calibration Curves for Splitters—New System

Splitter inlet flow was 9 ml/min and outlet flow was measured with the electronic flowmeter. With the control valve of a splitter fully closed (all incoming air passing through the restrictor needle), the manometer reading was about 30 cm. Manometer readings and corresponding flow rates were then recorded for all manometer readings between 1 and 30 cm at 1-cm increments, and between 1 cm and −0.2 cm, which corresponded to a fully open valve, at 0.2-cm increments. The procedure was repeated for all four splitters, and the data were used to prepare calibration curves.

Measurement of Volatile Delivery Rates by SPME

With sample splitters and makeup air set to the desired levels, the sample line to the wind tunnel was separated at the junction of the 4-mm- and 3-mm-ID sections (about 30 cm from the mixing zone, Figure 1), and an SPME fiber was inserted about 3 cm into the tubing through which the sample was emerging. The SPME device was clamped in place so that the fiber did not touch the tubing walls. The fiber was 100 μm poly(dimethylsiloxane) (Supelco) and sampling time was 30 min. After the collection period, the fiber was withdrawn into the protective needle and the sample was analyzed by gas chromatography. Desorption time was 30 sec.

The Hewlett-Packard 5890 Series II GC was equipped with a 30-m DB-1 capillary column (0.32 mm ID, 5 μm film, J&W Scientific, Folsom, California), cool on-column injector, flame ionization detector, and integrator. A 10-cm retention gap made from 0.53 mm ID deactivated fused silica tubing was attached between the inlet and column so that the diameter was large enough to accommodate the SPME fiber. Temperature program was 50°C for 1 min, then 10°C/min to 250°C, followed by a 6-min hold at 250°C. Injector temperature was 200°C, and the detector temperature was 250°C. Carrier gas was helium.

The volatile delivery rate for compound n was calculated from the GC-SPME peak area according to:

$$D_n = (A_n \times R_n \times F)/K_n$$

where D_n was the delivery rate of compound n into the wind tunnel (in nanograms per second), A_n was the integrator GC peak area for compound n from the SPME injection, R_n was the GC-FID response factor for compound n (in nanograms per area unit), K_n was the SPME calibration factor for compound n (per milliliter), and F was the measured total airflow rate past the SPME fiber

(in milliliters per second) when the sample was collected. R_n is a property of the GC detector and integrator and is the proportionality constant between mass of analyte and peak area. R_n is used in any calculations of analyte amount from peak area, not only with SPME, and is readily measured by injecting a known volume of a quantitative standard solution into the GC. On the other hand, K_n is a property of the SPME fiber. SPME is an equilibrium process, and at equilibrium, the amount of analyte in the fiber coating (in nanograms) is directly proportional to the concentration in the gas phase (in nanograms per milliliter). The calibration factor K_n is the proportionality constant. K values have been determined, or can be predicted, for a wide range of compounds (Bartelt, 1997). The value, $(A_n \times R_n)/K_n$, represents the concentration of the analyte in the system tubing (in nanograms per milliliter). Multiplication by F converts this value into the rate of mass flow out of the end of the tubing. The above calculations can be done automatically for the key peaks of each GC run if the integrator or data system allows user-prepared macro programs to be run.

The success of the quantitation method depends on the commercial fibers being sufficiently uniform so that the published calibration factors are valid for any individual fiber of that type. In our experience this is reasonable, and Martos and Pawliszyn (1997) have a similar expectation. This method also depends on the volatile delivery rate from the sample being constant over the entire (30-min) sampling period. This is usually reasonable and can be verified by taking a series of samples. Finally, the method depends on the SPME fiber equilibrating with the gas-phase analytes. A sampling time of 30 min is sufficient for the compounds used in this study (Bartelt, 1997), but a longer sampling time might have to be employed for those of lower volatility.

Verification of Splitter Performance with Chemicals—New System

Two volatile sources were prepared. One was aqueous ethanol (27 g, diluted to 100 ml with water). This was added to a 125-ml Erlenmeyer flask containing a magnetic stirring bar. The inlet airstream swept through the headspace of the flask but did not bubble through the solution (see Figure 1). Stirring promoted equilibration of the volatiles between aqueous and gas phases. The second volatile source was a rubber septum to which 5 mg ethylguaiacol (4-ethyl-2-methoxyphenol, Lancaster) was added, and the septum was placed in the linear sample tube. Ethylguaiacol is an attractant for *C. humeralis* (B.W.Z., unpublished data). Each volatile source was connected to one of the splitters. Inlet airflow rate was 9.0 ml/min for both volatile sources. Makeup airflow was 80.0 ml/min. Seven splitter flow rates were chosen, nominally 1%, 3%, 6%, 12%, 25%, 50%, and 100% of the inlet flow. Manometer readings were recorded at each flow rate. The order of testing for the seven flow rates was independently randomized for each volatile source, but during the experiment both volatile

sources were operated simultaneously. The volatile concentration in the outlet airstream was measured by SPME, and the delivery rate of each compound (in nanograms per second) was calculated from the total airflow (makeup plus flow from both splitters) and the SPME data. There were two replications for each of the seven splitter settings.

Beetles and Bioassays

The culture of *C. humeralis* was established from beetles collected near Fresno, California, and beetles were reared on an artificial diet (Dowd, 1987), modified as described by Bartelt et al. (1993).

The wind tunnel was stocked with about 1000 *C. humeralis* adults of mixed sex and about one week of age. As with other *Carpophilus* species, the beetles initially crawled into corners and became motionless. However, after 1–2 hr with the lights on and with the temperature at 28–30°C, the beetles began to fly about spontaneously. When about 100 were in the air at any instant bioassays could begin. Tests lasted 2 min from the time a volatile stream was directed into the wind tunnel, and the numbers of beetles alighting on the landing target were recorded. Typical behavior prior to landing was a slow, hovering, casting flight up to the target, beginning when a randomly flying beetle first encountered the odor plume. Beetle landing data were transformed to $\log(x + 1)$ before statistical analysis to stabilize variance.

Effect of Makeup Air on Bioassay Response—Prototype System

An aqueous solution of ethanol (2.2 g/liter), acetaldehyde (10.4 mg/liter), ethyl acetate (1.1 mg/liter), and 2-methyl-1-butanol (1.0 mg/liter) was prepared, and 400 ml were transferred to the 500-ml sample flask equipped with magnetic stirring bar. Air was passed through the sample flask headspace at 80 ml/min. The resulting volatile mixture was known to be highly attractive to the beetles (R.J.B., unpublished data). Headspace concentrations of components were measured by SPME-GC at the outflow of the splitter with the makeup air turned off.

For the bioassay experiment, splitter outflow levels were 100%, 10%, and 1% of maximum and off (control). For each splitter level, the makeup air was either on or off, giving a total of eight treatments. These were tested in random order, and four replications of the entire experiment were conducted. Beetle landings during a 2-min period were recorded, and there was a 1-min rest period between tests. For tests with the make-up air off, the splitter valve was initially fully closed so that 100% of the test volatiles entered the wind tunnel. As soon as the beetles began to respond, the splitter valve was adjusted to the proper test setting, and then the timer was started. This procedure served to quickly

fill the air line to the wind tunnel with the appropriate test mixture; with no makeup air and slow sample flows, filling of the air line would have been very slow.

Transformed bioassay data were subjected to analysis of variance as a 4 × 2 factorial design, considering replications of the experiment as blocks.

Bioassay Response Time for Changes in On/Off Valve—New System

An aqueous solution (100 ml) with 3-hydroxy-2-butanone (300 mg) and ethanol (290 mg) in a 125-ml flask was attached to the splitter inlet. Airflow through the flask headspace was 9 ml/min, and all of the sample effluent was directed toward the wind tunnel. Volatile delivery, measured by SPME, was 10 ng/sec for 3-hydroxy-2-butanone and 50 ng/sec for ethanol. This mixture was known to be highly attractive (B.W.Z., unpublished data). The bioassay began with flow directed to waste (V2 open, Figure 1). Total landings were recorded every 10 sec. Sample flow was directed to the wind tunnel (V2 closed) at time 120 sec and was directed back to waste at time 310 sec. The bioassay was replicated four times.

Bioassay Comparison of a Natural and Synthetic Attractant—Prototype System

Fermenting bread dough, an excellent attractant for nitidulid beetles (Lin and Phelan, 1991) was prepared with whole wheat flour, sugar, and water in a 3:2:3 ratio and inoculated with dried baker's yeast. About 5 g was transferred to a 10-ml vial with a 1.0-cm opening. After two days of fermentation at room temperature, the vial was placed upright in a 250-ml flask and the flask attached to the splitter system. Humid air was passed through at 80 ml/min with a bubbler installed in the air line before the sample flask.

After about 1 hr, the airstream was sampled by SPME for 30 min and the sample analyzed by GC. From the peak integrations and previous experience with the compounds, an aqueous mixture of seven volatiles was prepared within 1 hr that would approximate the volatile delivery rate of the natural sample when attached to the splitter (Table 2 below). The actual delivery rate from the synthetic mixture was then measured by SPME for confirmation.

The natural bread dough was bioassayed at eight splitter settings, starting with 100% of the culture volatiles being directed to the wind tunnel, followed by settings of 50%, 25%, 12%, 6%, 2.5%, 1%, and 0.5%. Tests lasted 2 min and were separated by 1 min. The process was repeated with the synthetic mixture. Then a second replication was obtained for each treatment. The entire experiment was repeated on a second day, except that the order of testing delivery rates was lowest to highest. The transformed bioassay data were subjected to analysis of variance, as an 8 × 2 factorial design (eight doses and two sources), considering the four replications of the experiment as blocks.

Three-Component Mixture—New System

Aqueous solutions were prepared for ethanol (0.27 g/ml), acetaldehyde (0.90 mg/ml), and ethyl acetate (0.55 mg/ml). Each solution (100 ml) was added to a 125-ml flask and the flask connected to a splitter. Airflow through each sample flask headspace was 9.0 ml/min, and makeup air was set at 80.0 ml/min. With the splitter valves fully closed, so that all of the effluent emerged from the splitters, delivery rates were 5000 ng/sec for ethanol, 250 ng/sec for acetaldehyde, and 380 ng/sec for ethyl acetate. These levels of ethanol and acetaldehyde simulated production by a very attractive 50-g sample of bread dough. The ethyl acetate level was higher than from the dough but was similar to certain fungal cultures (R.J.B., unpublished data).

For each compound four delivery rates were tested: 100%, 10%, and 1% of the maximum rate and none (the control). These levels of the three compounds were tested in all possible combinations (resulting in a $4 \times 4 \times 4$ factorial design with 64 different treatments). A single treatment was presented in the wind tunnel for 2 min, and the number of landings was recorded. This was followed by a 1-min pause during which the splitters were reset and no effluent entered the wind tunnel. The order of testing the treatments was formally randomized, and the 64 two-minute tests were completed in about 3 hr on one bioassay day. The entire experiment was replicated three times, and replications were considered as blocks in the analysis.

Because the level of beetle activity in the wind tunnel does not remain strictly uniform during an experiment as long as 3 hr, analysis of covariance was used to allow for changes in beetle activity over time. At the beginning of a typical experiment (2–3 hr after loading the wind tunnel), landings at an attractive bait (e.g., bread dough scent) were about 100/2-min test. Responses normally increased further by 50–100% and then gradually subsided again during the subsequent 3–4 hr. The analysis model included both linear and quadratic terms so that the correction for changes in beetle activity could have a realistic curvature. A separate correction curve was allowed for each of the three bioassay days (accounting for six degrees of freedom). Adjusting for the covariates in the analysis had almost no impact on the estimated main effects and interactions for the three chemicals, but it did reduce residual error and increased the precision of comparisons.

RESULTS

Splitter Calibration and Performance

An example calibration curve is presented in Figure 3 (top panel). The curve demonstrates that the relationship between the rate of airflow through the

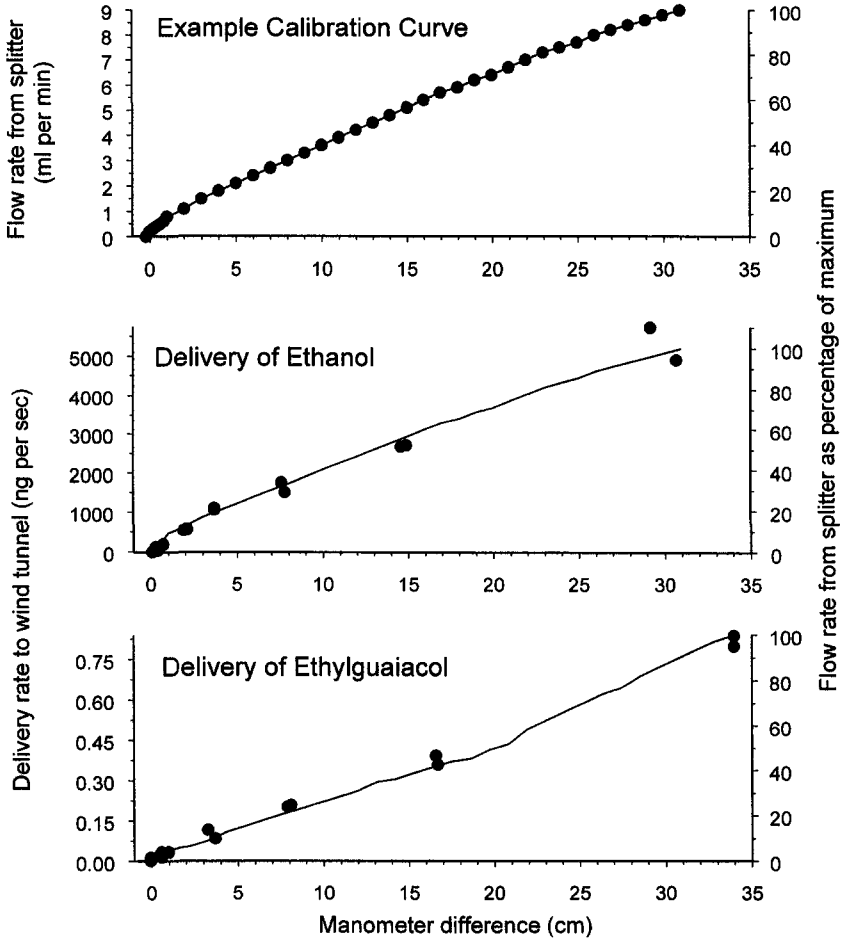

FIG. 3. Relationships between airflow through the restrictor needle and manometer reading (upper panel) and between delivery rate of chemicals and manometer reading (data points in lower two panels, relative to left-hand axis). Delivery rates were measured by SPME (see text). In the lower two panels, the appropriate splitter-flow/manometer calibration curves (solid lines, relative to right-hand axis) were superimposed on the SPME data to demonstrate the correspondence between splitter flow rate and chemical delivery.

splitter needle and the pressure reading on the manometer is essentially linear. Delivery of ethanol from an aqueous solution and delivery of ethylguaiacol from a rubber septum (as measured by SPME) corresponded nicely to the splitter calibrations determined with flow rates (Figure 3, lower panels).

TABLE 1. EFFECT OF MAKEUP AIR ON TOTAL FLOW RATES, SAMPLE CONCENTRATIONS, AND BIOASSAY RESPONSES

Volatile level (% of sample maximum)	Airflow (ml/min)			Ethanol		Mean bioassay landings ($N = 4$)[a]
	From sample	Makeup	Total	Final conc. (ng/ml)	Delivery rate (ng/sec)	
100	80	80	160	230	600	226.6a
100	80	0	80	450	600	212.3a
10	8	80	88	41	60	14.3b
10	8	0	8	450	60	15.5b
1	0.8	80	80.8	4	6	0.0c
1	0.8	0	0.8	450	6	0.7c
Control	0	80	80	0	0	0.2c
Control	0	0	0	0	0	0.3c

[a]Means followed by the same letter were not significantly different [LSD, 0.05 level, analysis in log $(x + 1)$ scale].

Effect of Makeup Air on Bioassay Response

Airflow rates, chemical concentrations in the airstream, chemical delivery rates, and bioassay responses are summarized in Table 1. The presence or absence of makeup air did not affect the bioassay response ($F = 0.41$, $P = 0.53$, $df = 1, 21$), but the effect of splitter setting was very clear ($F = 254$, $P = 0.0000$, $df = 3,21$). There was no interaction between effect of makeup air and splitter setting ($F = 0.14$, $P = 0.93$, $df = 3,21$).

Timing of Bioassay Responses When On/Off Valve is Switched

The lag time between closing the on/off valve and first emergence of the volatile stream into the wind tunnel was 10 sec, calculated from tubing volumes and flow rates. No behavioral response was expected within 10 sec of closing the valve, and none was observed (Figure 4). Beetles began to land with slightly increased frequency as time approached 20 sec, and by 30 sec, landings had reached the steady-state response level (about eight landings per 10 sec). Response dropped back to the control level almost instantly when the flow to the wind tunnel was stopped.

Bioassay Comparison of Natural and Synthetic Bread Dough

Dose–response curves for fermenting bread dough volatiles and for the synthetic approximation are shown in Figure 5. Maximum response was about

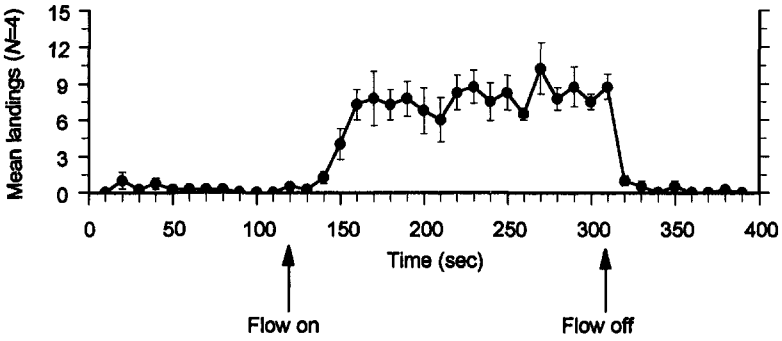

FIG. 4. Landing rate per 10-sec interval for *C. humeralis*. Flow of attractant was turned on and then off again during the test period, as indicated by arrows. Attractant blend included 3-hydroxy-2-butanone and ethanol. Error bars are standard errors.

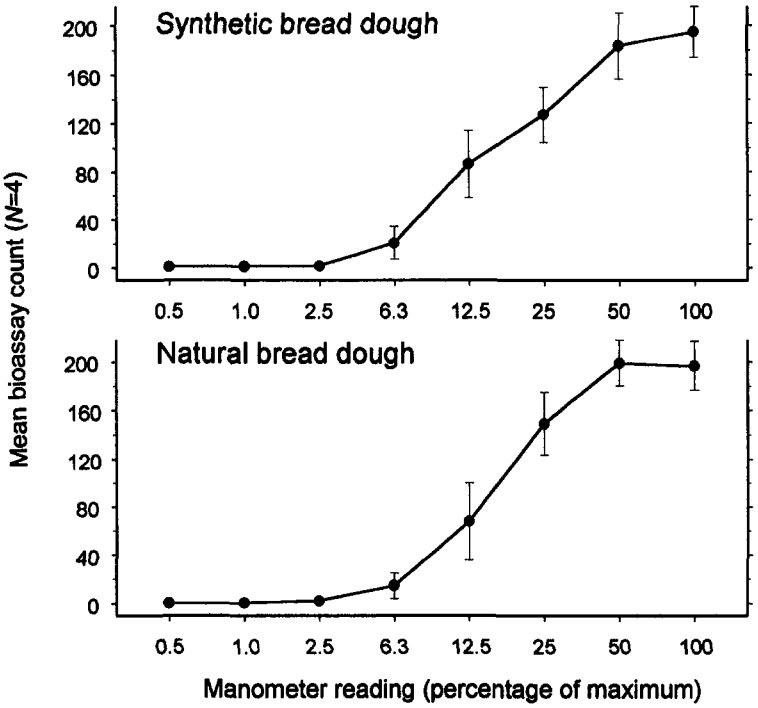

FIG. 5. Responses of *C. humeralis* to synthetic and natural bread-dough volatiles at a range of attractant levels. Error bars represent standard errors.

TABLE 2. MEASUREMENT OF VOLATILE-DELIVERY RATE BY SPME FOR SAMPLE OF BREAD DOUGH AND FOR SYNTHETIC MIXTURE INTENDED TO APPROXIMATE BREAD DOUGH

Compound	Delivery rate		Composition of synthetic mix (mg/l of water)
	From bread dough (ng/sec)	From synthetic mix (ng/sec)	
Acetaldehyde	50	38	10.4
Ethanol	380	410	1300
1-Propanol	1.1	1.3	2.2
Ethyl acetate	0.83	trace[a]	0.11
Isobutanol	1.7	2.3	2.5
3-Methyl-1-butanol	1.9	2.8	2.6
2-Methyl-1-butanol	0.70	1.2	1.0

[a]GC peak was visible but not integrated; 0.83 ng/sec of ethyl acetate from dough was very near the limit of detection.

200 landings per 2-min test, and there were almost no landings on the volatile-free control. There was no evidence that the two curves do not coincide, and the natural and synthetic baits performed equivalently. For both baits, the lowest level with greater than control activity was 6% of the maximum (Figure 5). This corresponded to an ethanol level of 23–25 ng/sec, with the other components proportionally reduced also (Table 2).

Bioassay Analysis of Effects of Ethanol, Acetaldehyde, and Ethyl Acetate

Mean responses varied from over 100 per test with the highest levels of the three compounds to less than 1 per test with controls. The analysis of covariance (Table 3) indicated that all three compounds had highly significant effects. The interactions between acetaldehyde and ethanol or ethyl acetate were relatively small, and the effect of acetaldehyde was nearly independent of how much, or what mixture, of the other compounds was present. In Figure 6 (top panel), this is illustrated by two examples, acetaldehyde alone and with the high levels of both ethanol and ethyl acetate. The gradual increase in response with rising acetaldehyde level is evident in both cases. Because of the logarithmic data transformation, the graph represents a multiplicative relationship, and the multiplicative relationship is essentially constant regardless of which other treatments were with acetaldehyde. The 25- and 250-ng/sec levels of the compound correspond to increases in landings of 2.0× and 4.7×, respectively, relative to

TABLE 3. ANALYSIS OF COVARIANCE FOR DOSE EFFECTS AND INTERACTIONS OF ETHANOL, ACETALDEHYDE, AND ETHYL ACETATE[a]

Source	*df*	SS	MS	*F*	*P*
Ethanol (EtOH)	3	14.116	4.705	94.0	<0.001
Acetaldehyde (AcAld)	3	12.459	4.153	82.9	<0.001
Ethyl acetate (EtAc)	3	14.415	4.805	95.9	<0.001
Day	2	25.678	12.839	256.3	<0.001
EtOH × AcALD	9	0.900	0.100	2.00	0.045
EtOH × EtAc	9	6.010	0.668	13.3	<0.001
AcALD × EtAc	9	0.770	0.086	1.71	0.094
EtOH × AcALD × EtAc	27	1.173	0.043	0.87	0.656
Residual	120	6.010	0.050		
Total	185	81.531			
Summary for covariates:					
Residual (ignoring covariates)	126	15.629			
Covariates	6	9.619	1.603	32.0	<0.001
Residual (adjusted for covariates)	120	6.010	0.050		

[a] Data transformed to log $(x + 1)$.

when acetaldehyde was absent, but the increase for the 2.5-ng/sec level was not significant.

The relationship between ethanol and ethyl acetate was more complex, as indicated by the highly significant interaction involving these compounds in Table 3. As shown in Figure 6 (lower panel), the effects of these compounds were not multiplicative. Rather, to a first approximation, the net effect of any combination was equal to the greater of the two individual effects. In addition, at the highest level of ethyl acetate (380 ng/sec), the response in combination with ethanol at 500 ng/sec was significantly less ($P < 0.01$) than that in combination with any other level of ethanol (Figure 6, lower panel, left-hand cluster of bars). Nevertheless, either ethanol or ethyl acetate, by itself, showed a strong relationship between amount and response. This is evident for ethanol in the right-hand cluster of bars in Figure 6, lower panel, and for ethyl acetate, in the white bars of all clusters in the lower panel. Alone, either ethanol or ethyl acetate could increase the response by a factor of as much as 26, but combining these compounds gave no further increase. Alone, the threshold level for ethanol was between 50 and 500 ng/sec, and for ethyl acetate, the threshold was less than 3.8 ng/sec. For these two compounds, the threshold for either one clearly depends on how much of the other is present.

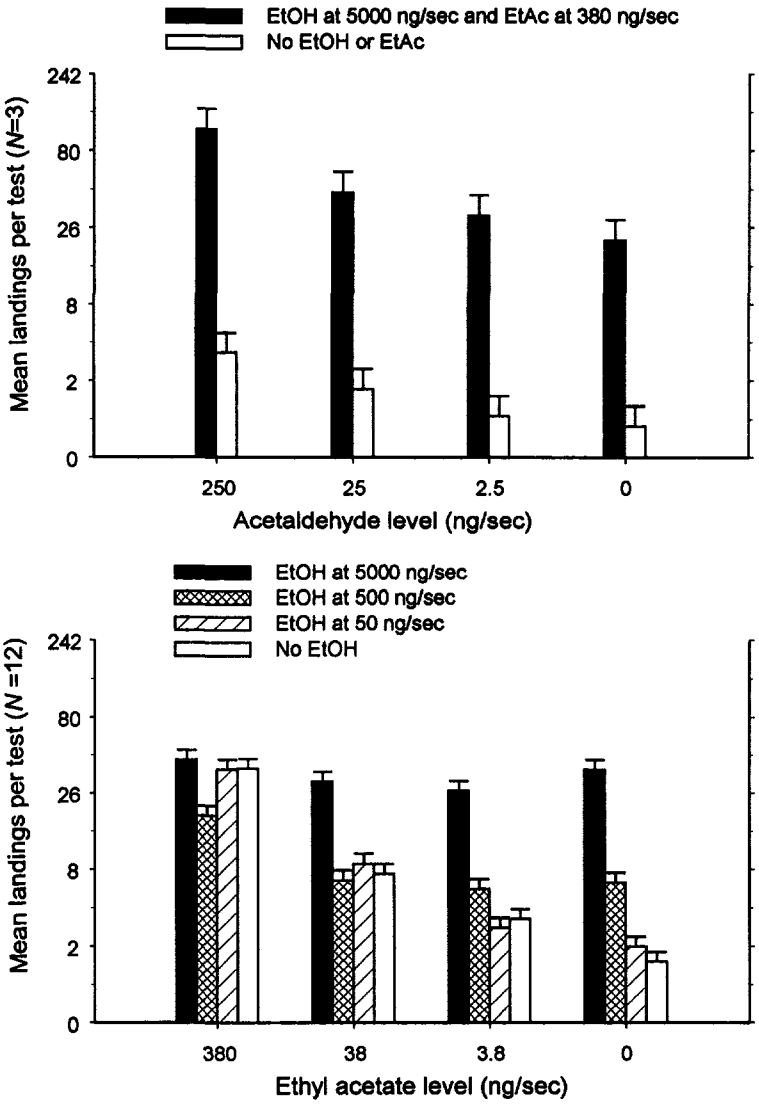

FIG. 6. Responses of *C. humeralis* to various combinations of ethanol, acetaldehyde, and ethyl acetate. Upper panel: Dose response for acetaldehyde when the other volatile levels are fixed (two examples). Each bar is a mean over the three days of the experiment. Lower panel: Interactions between ethanol and ethyl acetate. Each bar is a mean over the three days of the experiment and all four acetaldehyde levels. Error bars are standard errors from the analysis of covariance. The y axis was scaled to preserve the relationships among treatments existing in the $\log(x + 1)$ analysis scale.

DISCUSSION

Advantages of Splitter System

The system described here has certain practical advantages. The test volatiles pass through a short, low-volume path lined with inert and nonadsorbent materials. The volatiles reaching the bioassay arena do not pass through any valves or control devices that could selectively remove sample components or introduce contaminants into the airstream. The entire system is easily disassembled for cleaning, if needed. The system can be constructed from readily obtained and inexpensive materials. The simplicity of design and operation make it reliable and free from frequent or tedious maintenance or adjustment.

Quantitation of Volatiles

With SPME it is possible to measure the concentration (and therefore the delivery rate) of volatiles emerging from the splitter system. This measurement can be done rapidly (the SPME device equilibrates with many compounds within 30 min) and without disrupting the sample setup in any way. The SPME method does not use solvents, so sample components cannot be hidden under a solvent GC peak. SPME can operate over a large range of volatile delivery rates. In this study, delivery rates included 50–5000 ng/sec for ethanol and 0.008–0.8 ng/sec for ethylguaiacol. SPME is intrinsically more sensitive to less volatile compounds (Pawliszyn, 1997). This can be an advantage in insect behavioral research because highly active, heavier compounds such as pheromones must be measurable at very low levels. The sensitivity of SPME can be further increased about 10-fold by making the measurement with the flow of makeup air off. This prevents dilution of the volatile stream with air, and the increased concentration leads to an increased amount absorbed by the SPME fiber. A disadvantage is that it takes longer for the system to stabilize without makeup air. The splitter can further broaden the range in which known levels of chemicals are bioassayed. For example, if a sample delivers the minimum measurable by SPME, the splitter and its calibration curve would allow known levels to be bioassayed that are as much as two orders of magnitude below the SPME minimum.

Types of Volatile Samples

The system has great flexibility with respect to sample type for bioassays. Three examples were described: a living microbial culture (bread dough), aqueous solutions of volatile organic compounds, and a semivolatile organic compound adsorbed on rubber. Many other configurations are possible, including flasks with living insects on host material (e.g., for pheromone studies).

An advantage of this system for studying a living sample, such as a microbial culture, is that volatile production can be left to reach a steady state with a constant stream of air passing over it. Then, the exiting airstream, or any fraction of it, can be diverted into the bioassay arena without disrupting the living system or changing the airflow rate over it. It would even be feasible to house the sample container in a small incubator set near the splitter apparatus, so that temperature, light regime, or other parameters could be carefully controlled. It is also possible to humidify the air with a "bubbler" before passing it into the sample container to prevent sample desiccation.

Water is a good controlled-release medium for many organic compounds, especially those of higher volatility or polarity. Compounds of intermediate polarity such as esters and aldehydes will mix with water at low concentrations. In our experience, such solutions can usually cover the ranges of delivery rates that are of biological interest, and delivery from the solutions is quite uniform over hours or days. Furthermore, the physical chemistry of these aqueous solutions and their headspace properties are well known. For dilute solutions at equilibrium, the concentration of a compound in the headspace is proportional to its concentration in the aqueous phase (Henry's law), and the values of these proportionality constants are available for a huge variety of organic compounds (e.g., Betterton, 1992). Thus it is often possible to calculate from tables, in advance, the amount of compound to add to a flask of water that will generate a certain headspace concentration. Headspace concentrations when the air is flowing slowly seem to be quite similar to when it is static. In our experience, water vapor does not have a detectable influence on the behavior of *C. humeralis*, but it could affect other species. The appropriateness of water as a controlled release medium would have to be checked on a case-by-case basis.

Less volatile compounds can be delivered from other formulations such as rubber septa. We used a straight tube to hold such volatile sources, rather than a flask, so that flow eddies and large adsorptive surfaces could be avoided. In this study, rather polar semivolatile ethylguaiacol in a rubber septum was successfully delivered and controlled at low levels. Pheromones and other compounds of similar size would also be amenable to control by the splitter system.

Bioassays and Physical Measurements

The expected proportionality between splitter flow rate and manometer reading was confirmed, and manometer calibration curves were developed. Based on measurements by SPME, the actual delivery of volatiles was similarly related to manometer reading.

Bioassay experiments supported the physical measurements of system characteristics. Closing or opening of the on/off valve (V2), which directed sample flow into the wind tunnel or to waste, was rapidly reflected in beetle responses

Using bread dough scent, we also demonstrated that proper adjustment of the control valve (V1), guided by manometer readings, could provide dose–response data of the expected sigmoid form.

A bioassay experiment also verified that adding makeup air to the volatile stream does not affect beetle responses adversely. Using makeup air (80 ml/min) with the splitter system is desirable because it allows any adjustments of the splitters to be rapidly transmitted to the wind tunnel, and it also allows constant purging of system tubing so that residues of test volatiles are less likely to accumulate. However, makeup air also dilutes volatiles emerging from a splitter needle. From the experiment, the weight of compound entering the wind tunnel per unit time is the key quantity. Within experimental limits, a dilute sample entering rapidly would have the same effect as a concentrated sample entering slowly. Therefore, behaviorally relevant units for expressing volatile delivery would be nanograms per second.

The maximum linear flow rate of samples plus air entering the wind tunnel through the 2-mm-ID glass nozzle is only slightly faster than the bulk flow of air through the wind tunnel. Therefore, the sample stream should not cause unusual eddies or other undesirable flow characteristics for any of the experimental flow rates; rather, the sample stream should be rapidly entrained in the wind-tunnel flow. Intuitively, the actual weight of compound entering the wind tunnel per unit time should be more important behaviorally than the amount of air it is mixed with before entering the wind tunnel, and this is what was found.

An important question in host-volatile studies is whether a synthetic mixture fully accounts for the activity of a natural source. Determining this can be very difficult because natural volatile sources are often ephemeral and change rapidly. The system described above can be very helpful in such instances. In this study, fermenting bread dough was used as an example natural source. The steady-state volatile emission rate was measured quickly by SPME, and within an hour of that measurement, an aqueous synthetic mixture was prepared that would deliver the key volatiles at similar rates; this was quickly verified by SPME. Dose–response bioassay data were then obtained for both the natural and synthetic volatile sources, without further sample manipulation. In this example, the dose–response curves for the natural and synthetic samples were nearly identical. Finally, the experiment provided a good measure of the absolute threshold level for the bread dough volatiles, above which significant attraction would occur.

The value of multiple splitters was demonstrated with the comparison among various levels of ethanol, acetaldehyde, and ethyl acetate. In this study a fairly complex experimental design was executed rather quickly and simply with the apparatus. Bioassay amounts for all three compounds covered two orders of magnitude, and all possible combinations of these were tested. Having 64 different treatments would have presented a formidable task if sample handling

had involved anything more complicated than setting three valves to desired manometer readings.

The biological effects of ethanol, acetaldehyde, and ethyl acetate were examined previously (Lin and Phelan, 1991), although their study involved a different nitidulid and also included additional compounds. Their conclusions were that acetaldehyde and ethyl acetate both contributed to attraction but that ethyl acetate was interchangeable with ethanol. Essentially the same general conclusions were drawn here, but because of the new equipment, these could now be stated with greater precision (i.e., in terms of threshold amounts, multiplicative relationships, and dose responses). This was possible because absolute amounts of chemicals experienced by the beetles were now measurable and because including compound dose as an experimental factor did not increase the number of solutions to be prepared or otherwise complicate the handling of samples.

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WHICH CHEMICAL CONSTITUENTS FROM DOG FECES ARE INVOLVED IN ITS FOOD REPELLENT EFFECT IN SHEEP?

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Abstract—This study is an attempt to identify the active chemicals (signal) of the odor of dog feces that suppress feeding in domestic sheep. The repellent effects of the odors of dog, wolf, pig, and sheep feces (potential predator and nonpredator species) were assessed on sheep, using a food-choice situation. The odors of wolf and dog feces had the highest repellent effect. A total pentane extract of dog feces was split by micropreparative gas chromatography and the fractions obtained were analyzed and presented to sheep in a food-choice situation. The quantitatively major constituents of the pentane extract, identified by gas chromatography–mass spectrometry, are indole and fatty acids. In food repellency tests, indole had no repellent effect. The active odorous signal contained in dog feces appears to consist of fatty acids mixed with neutral compounds acting synergically. These experiments underline the complexity of this biological signal and constitute a first step in the development of a practical repellent for ungulates.

Key Words—Sheep, fecal odor, volatile constituents, domestic dog, *Canis familiaris*, wolf, repellency, interspecific communication.

INTRODUCTION

Ungulates, as well as rodents, inflict feeding damage to fruit and forest trees and to agricultural crops. The use of chemicals that deter browsing or barking

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by ungulates could help reduce damage to this vegetation. The odor of excretions (urine, feces) and secretions (from anal glands) of predators have been reported to induce feeding avoidance in animals that are potential prey (for reviews see Weldon, 1990; Müller-Schwarze, 1990). Compounds from the anal glands of various Mustelidae have been reported to reduce feeding damage by rodents and lagomorphs (for review see Weldon, 1990). For example, Sullivan and his colleagues have used isolated sulphur-containing compounds previously identified mainly in the anal gland secretions of Mustelidae (for review see Müller-Schwarze, 1990). Feces from Felidae or Canidae, or urine from Canidae reduce feeding damage by several species of deer (*Odocoileus* spp. and *Cervus elaphus*) (Abbott et al., 1990; Swihart et al., 1991 for review see Weldon, 1990). These same fecal odors are also repellent for sheep (*Ovis aries*) (Arnould and Signoret, 1993; Pfister et al., 1990), cattle (*Bos taurus*) (Pfister et al., 1990), and goat (*Capra hircus*) (Weldon et al., 1993).

The authors of most of those studies have not attempted to identify the active components of the crude biological products. Such an identification could contribute to the development of practical repellents designed to protect agricultural crops or plantations. Two studies, however, do report on the chemicals possibly involved. First, trimethylthiazoline induces in laboratory rats (*Rattus norvegicus*) a fright reaction similar to their response to fox feces from which it has been extracted (Vernet-Maury, 1980). Secondly, Abbott et al. (1990) have demonstrated that a mixture of fatty acids, alcohols, and nitrogenous compounds identified in African lion feces mimics the food repellent effect of the crude feces on red deer (*Cervus elaphus*).

Previously we have observed that the odor of dog feces is an especially efficient repellent in a controlled food choice situation with sheep (Arnould and Signoret, 1993), as well as in a much more natural foraging situation (pasture) in sheep, roedeer (*Capreolus capreolus*), and red deer (Arnould et al., 1993). The present study is designed to identify the active chemicals of dog fecal odor that function as repellent for sheep, using physicochemical methods and behavioral tests. First, the food repellent effect of the odor of the fecal matter from various species was compared with their volatile constituents (extracted in pentane). In a second series of experiments, the volatile constituents of a pentane extract of dog feces were in part identified by gas chromatography–mass spectrometry (GC-MS). These volatile constituents were fractionated or chemically modified and tested together with single compounds to determine those that were active as food repellents.

METHODS AND MATERIALS

Animals

Thirty ovariectomized Ile-de-France ewes (3–8 years old) were tested. They were kept indoors and fed daily 650 g of a mixture of maize, wheat, dehydrated

alfalfa, a complement of vitamins and minerals, and straw *ad libitum*. Prior to the beginning of an experiment, the animals were fasted for a minimum of 20 hr. They were fed, as usual, once a day, but just after the end of the experiment.

Products Tested

In experiment 1, the food repellent effect of biological substances (feces from domestic dog, wolf, pig, and sheep) and of pentane extracts of domestic dog, wolf, and pig feces were tested. In experiment 2, we tested fractions and single compounds (indole, quinoline) from the extract of dog feces. To determine the repellent threshold of dog feces extract, sheep were tested with several dilutions of the pentane extract (1/2000 to pure extract). All fractions of the extract were then tested on animals at the dilution corresponding to this threshold. Dog fecal matter was collected from a single animal (adult female Epagneul Breton breed). The fecal matter of the other species was collected from several animals. The feces were frozen within 90 min in the case of dog, pig, and sheep or within about 48 hr for wolves. All of these samples, as well as the pentane extracts, were stored at $-18°C$. All animals were fed with commercial food, except for the wolves which, were fed different kinds of raw meat.

Behavioral Tests

The effectiveness of the products as repellents was measured using a food choice test (Arnould and Signoret, 1993). Each animal was individually tested for 3 min in a pen (3.8×2 m) following 1 min isolation in a starting box. The testing pen contained two stainless steel troughs (control and experimental), each with a screen-bottomed bin (20×20 cm) that contained 30 g of maize. Odorous products were placed in the experimental trough, below the food bin. Feces (about 35 g) were placed 4 cm under the screen, and solutions of pentane extracts or single compounds (50 μl) were deposited on filter paper placed 2 cm below the screen (and changed for each animal). The amount of maize eaten from each trough was recorded. To measure the repellent effect of an odor, we used either the reduction in the amount of maize eaten in the presence of the odor (experimental trough) when compared with the control trough or the amount of maize eaten in the presence of the odor when compared with another odor (comparison of the repellent effect of two odors).

The animals used in these experiments were very familiar with the test situation. They were very quiet and highly motivated for food: their maximum feeding latency in a control situation (no odorous substance tested) was 2 sec. The absence of any neophobic reaction in all of these animals to novel odors was verified before the beginning of the experiments using amyl acetate [the method used has been previously described in Arnould and Signoret (1993)]. An odor has to be strongly repulsive to prevent feeding in the experimental situation.

Experimental Schedule

Several experimental series were conducted to allow us to compare the relative effects of different products. For example, in series 1 we tested feces from different species and pentane extracts of these feces. In series 2 we tested the total pentane extract of dog feces and fractions of this extract, etc., until we had a complete set of data. All products of the same series were tested on the same ewes. The order of products tested in a series was chosen at random.

The feces were tested twice on each animal, with the positions of the experimental and control troughs reversed between the two tests. On the other hand, the pentane extracts were tested only once on each of the ewes, because of the small volume of these extracts available (see below). In this case the position of the experimental trough was reversed for half the animals.

When an extract was to be modified (separated into fractions, methylation, etc.), an aliquot was put aside ahead of time to be used as a control in the behavioral tests.

Extraction and Separation of Volatile Chemicals from Pentane Extracts

Extraction of Volatile Chemicals. The volatile compounds in the feces were extracted in a Likens-Nickerson apparatus by simultaneous steam distillation–extraction (SDE), according to the method described by Schultz et al. (1977). Fecal matter (200–300 g) mixed with 1 liter of water was extracted with 50 ml of pentane 99% (time of SDE: 2 hr). Pentane extracts obtained were concentrated in a Kuderna-Danish apparatus to 2 ml.

Extract Composition. The major volatiles from fecal matter were identified using a Girdel 32 gas chromatograph (Ross injector: 240°C) coupled to a Nermag R10-10C quadrupole mass spectrometer. The gas chromatograph was equipped with a fused capillary DB-Wax column (J & W Scientific) (25 m, 0.32 mm ID) with temperature programmed from 60°C to 240°C at 3°C/min. The carrier gas used was helium at a 0.3 bar pressure. Identification of natural compounds was achieved by comparison of their electron impact (EI) mass spectra with EI mass spectra from the EPA/NIH library of the SIDAR acquisition data system. Confirmation of molecular weights was obtained by chemical ionization using ammonia (NH_3) as the reactant gas. Comparisons of spectral data and retention times were made with commercial compounds when available.

Compositions of dog feces extracts and fractions were analyzed by gas chromatography, using two types of apparatus and conditions: (1) a Girdel 300 chromatograph (Ross injector and FID heated at 240°C) fitted with a fused capillary column DB-Wax (J & W Scientific) (25 m, 0.32 mm ID) with a programming temperature from 60°C to 240°C at 5°C/min, carrier gas was helium at 0.5 bar pressure and (2) Carlo Erba Fractovap 2900 chromatograph (splitless injector and FID: 240°C) fitted with a fused capillary column WCOT

FFAP-CB (Chrompack) (25 m, 0.32 mm ID); carrier gas was helium at 0.4 bar pressure, and temperature was programmed from 55°C to 120°C at 35°C/min, 5 min at 120°C, then 120°C to 240°C at 5°C/min.

Collection of Capillary Gas Chromatographic Fractions. Gas chromatographic separation of the pentane extracts was made using the method described by Malosse (1990). A Girdel 300 gas chromatograph (equipped with Ross injector heated at 240°C) was used. It was fitted with a wide-bore DB-1 column (J & W Scientific) (15 m, 0.53 mm ID), with temperature programmed from 100°C to 240°C at 10°C/min. The carrier gas used was helium at 0.3 bar pressure. Materials eluting from the capillary column were trapped at −80°C in a capillary collector and flushed with pentane. The total extract was split into fractions 1, 2, and 3. Components of the fractions were trapped until their peak heights were similar to those seen in the total extract.

Methylation of Pentane Extracts. A mixture of benzene (1 ml), methanol (2 ml), and H_2SO_4 36 N (four drops) was added to the total pentane extract (2 ml) and refluxed for 1 hr at 90°C. After cooling, this preparation was mixed with water to eliminate H_2SO_4 and methanol from the organic phase.

Hydrogenation of Pentane Extracts. Hydrogenation was performed with a catalyst (platinum IV) and hydrogen bubbling in the pentane extract for 5 min.

Preparation of a Synthetic Mixture. A synthetic mixture of 13 compounds was prepared according to quantitative data obtained by gas chromatographic analysis of the total extract of dog feces. This mixture included 10 fatty acids that were identified in this extract, indole, and two unidentified compounds trapped by micropreparative gas chromatography.

Statistical Analysis

Mean amounts of food eaten were compared using a Wilcoxon signed-ranks test for dependent samples.

RESULTS

Experiment 1: Comparative Effect of Fecal Odors and Pentane Extracts

All four crude feces samples and two of the three pentane extracts containing volatile constituents from these feces had significant repellent effects ($P \leq 0.05$ or ≤ 0.01) (Table 1). The repellent effect of the extract of pig feces bordered on statistical significance ($P = 0.06$).

To compare the relative repellent potency of these odors, the mean weights of maize eaten in the experimental trough were compared across the test odors. There was no significant difference in the repellent effects of wolf and dog feces. Pig feces were significantly less repellent than the former two feces ($P < 0.05$ in both cases). The difference observed between the repellent effects of sheep

TABLE 1. REPELLENT EFFECTS OF FECES AND PENTANE EXTRACTS ON CONSUMPTION OF FOOD BY 8 EWES FROM CONTROL AND EXPERIMENTAL TROUGHS, EACH CONTAINING 30 g OF MAIZE

	Mean weight of maize eaten (g ± SD)		
	Control trough	Experimental trough	P^a
Sheep feces	30 ± 00	12 ± 12	≤0.05
Pig feces	30 ± 00	19 ± 14	≤0.05
Dog feces	30 ± 00	3 ± 05	≤0.01
Wolf feces	30 ± 00	0	≤0.01
Extract of			
Pig feces	30 ± 00	17 ± 15	=0.06
Dog feces	30 ± 00	1 ± 04	≤0.01
Wolf feces	30 ± 00	1 ± 02	≤0.01

[a]Wilcoxon test.

and wolf feces was statistically significant ($P < 0.05$), but that between sheep and dog feces was not ($P > 0.05$). Similar results were obtained with the pentane extracts. The repellent effects measured in the presence of the pentane extracts of dog and wolf feces were not statistically different ($P > 0.05$). There was a significant difference between the repellent effects of the pentane extracts of pig and wolf feces ($P < 0.05$). The difference between pig and dog feces bordered on statistical significance ($P = 0.06$).

Experiment 2: Analysis of Active Repellent Components of Dog Feces Extract

Results from experiment 1 indicate that the odors of dog and wolf feces have a high repellent effect compared with the feces from the other species (i.e., pig or sheep). The pentane extracts of these feces had a similar effect.

Chemical Study of Odor of Dog Feces. GC-MS analysis was performed for one pentane extract of dog feces found to be representative of all the samples extracted. In the chromatogram of this extract (Figure 1), approximately 80 compounds were identified (Table 2). One of the most prominent compounds was indole. Numerous fatty acids with straight chains from C_4 to C_{18} were identified. Most of these were saturated acids. Esters, aldehydes, and heterocyclic compounds (including a sulphur-containing compound) were also identified.

Analysis of Repellent Effects of Three Fractions Derived from Pentane Extract of Dog Feces. Among the three fractions trapped by micropreparative

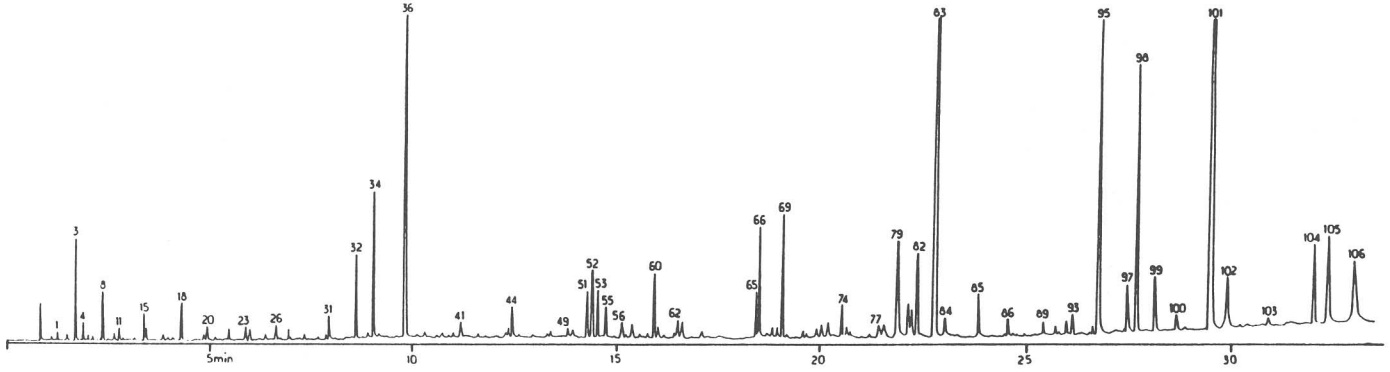

FIG. 1. Chromatogram of a total extract of dog feces obtained by GC-MS (see Methods and Materials).

TABLE 2. MAIN CONSTITUENTS IDENTIFIED IN EXTRACT OF DOG FECES

1 3-Methylbutanal
2 Propanoic acid propyl ester[a]
3 Hexanal[a]
4 Propanoic acid 2-methyl propyl ester[a]
5 Propanoic acid butyl ester[a]
6 Pentanoic acid propyl ester[a]
7 2-Heptanone[a]
8 Heptanal[a]
9 Pentanol[a]
10 Butanoic acid 1-methyl propyl ester[a]
11 2-Pentylfuran
12 2-Heptenal[a]
13 Butanoic acid 3-methyl butyl ester[a]
14 Butanoic acid pentyl ester[a]
15 4-Methyl 2-heptanone
16 Octanal[a]
17 Butanoic acid 3-methyl, 3-methyl butyl ester
18 Hexanol[a]
19 2-Nonanone[a]
20 Nonanal[a]
21 3-Octene 2-one[a]
22 2-Octenal[a]
23 1-Nonene 3-ol
24 Heptanol[a]
25 2-Nonene 4-one
26 2-Ethylhexanol
27 2-Decanone[a]
28 Decanal[a]
29 3-Nonene 2-one
30 Benzaldehyde[a]
31 2-Methylpropanoic acid[a]
32 2-Undecanone[a]
33 3,7-Dimethyl 6-octenal
34 Butanoic acid[a]
35 Phenylacetaldehyde[a]
36 3-Methylbutanoic acid[a]
37 2-Undecene 4-one
38 2-Dodecanone[a]
39 2-Propyl thiophene
40 2-Undecanol[a]
41 Pentanoic acid[a]
42 2,4-Decadienal[a]
43 NI[b]
44 2,4-Decadienal[a]
45 2-Tridecanone[a]
46 2-Dodecanol
47 2,4-Diterbutyl phenol
48 *cis*-Geranyl acetone[a]
49 Propanoic acid 2-methyl 2-phenyl ethyl ester
50 NI
51 Phenylethanol[a]
52 NI
53 Quinoline
54 2-Tetradecanone[a]
55 Tetradecanal[a]
56 1-Methylisoquinoline
57 NI
58 NI
59 NI
60 Phenol[a]
61 NI
62 2-Pentadecanone[a]
63 2-Tetradecanol
64 NI
65 2-Phenanthrenone 4,4a,9,10 tetrahydro 4a methyl
66 Hexadecanal[a]
67 Hexadecenal[a]
68 NI
69 Ethanone 1-(2-hydroxy 5-methyl phenyl)
70 NI
71 NI
72 2-Hexadecanone
73 NI
74 Heptadecenal
75 Decanoic acid[a]
76 Farnesol[a]
77 NI
78 Tricosane[a]
79 Octadecanal[a]
80 9-Octadecenal[a]
81 9-Octadecenal[a]
82 NI
83 1-H Indole[a]
84 Tetracosane[a]
85 Dodecanoic acid[a]
86 NI
87 NI
88 3′-4′-Dimethoxy acetophenone
89 NI
90 Tridecanoic acid[a]
91 NI
92 NI

TABLE 2. Continued

93 NI	100 NI
94 NI	101 Hexadecanoic acid[a]
95 Tetradecanoic acid[a]	102 Hexadecenoic acid[a]
96 Acid NI	103 Heptadecanoic acid[a]
97 Acid NI	104 Octadecanoic acid[a]
98 Acid NI	105 Octadecenoic acid[a]
99 Pentadecanoic acid[a]	106 Octadecadienoic acid[a]

[a]Compounds compared with commercial compounds (see Methods and Materials, Extract Composition).
[b]NI: not identified.

gas chromatography, only fraction 3 had a significant repulsion effect ($P < 0.05$) (Figure 2). A nonsignificant trend towards repulsion appeared with fractions 1 and 2 ($P = 0.07$ and $P = 0.06$) (Figure 2). There was no significant difference between the repellent effect of a mixture of fractions 1, 2, and 3 and the total extract ($P > 0.05$), indicating that no degradation of the active substances had resulted from the fractionation.

Fraction 3, the most active fraction, is composed mainly of linear fatty acids containing more than 12 carbon atoms. Its chromatogram and its identified constituents are described in Figure 3.

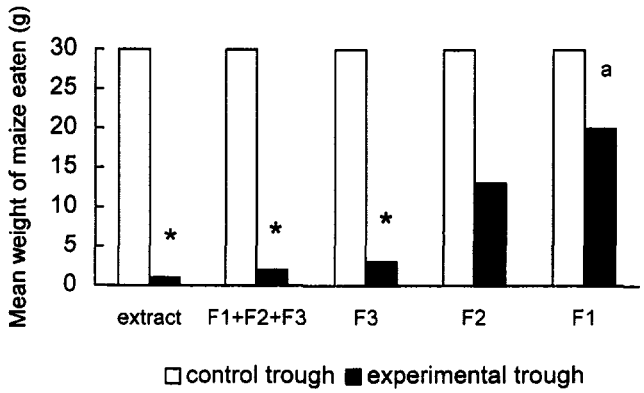

FIG. 2. Effect of the total extract from dog feces, fractions 1, 2 and 3 (F1, F2, F3, respectively) obtained by micropreparative gas chromatography from this extract, and a mixture of the three fractions (F1 + F2 + F3) on consumption of food by six ewes. *$P < 0.05$, [a]$P \leq 0.05$ *vs* all others (experimental trough).

56 1-Methylisoquinoline
66 Hexadecanal
75 Decanoic acid
79 Octadecanal
83 Indole
85 Dodecanoic acid
95 Tetradecanoic acid
97 Non identified acid
98 Non identified acid
99 Pentadecanoic acid
101 Hexadecanoic acid
102 Hexadecenoic acid
104 Octadecanoic acid
105 Octadecenoic acid
106 Octadecadienoic acid

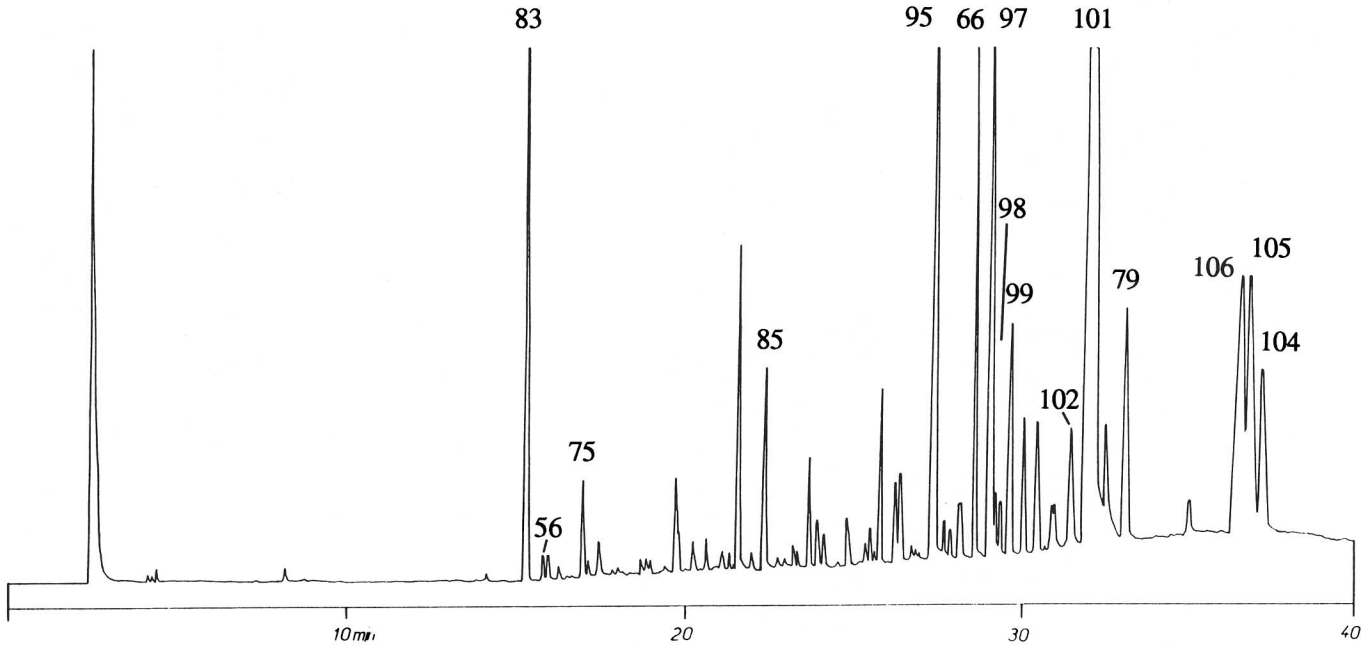

FIG. 3. Chromatogram of fraction 3 obtained from the total extract of dog feces (conditions described in *Collection of Capillary Gas Chromatographic Fractions* in Methods and Materials).

Fatty Acid Contribution to Repellent Effect of Dog Feces. The methylated (Figure 4A) and the hydrogenated (Figure 4B) extracts, had significant repellent effects ($P < 0.05$ in both cases) compared with the control. There was no significant difference in the weight of food eaten in the tests performed with the hydrogenated extract compared with the total extract (Figure 4B). With the methylated extract, a nonsignificant trend appeared when compared with the total extract ($P = 0.07$) (Figure 4A).

The synthetic mixture, containing mostly fatty acids and indole (Figure 5), had a significant repellent effect ($P \leq 0.05$) (Table 3), but this effect was significantly lower than that of the total extract ($P = 0.01$). Three of 10 ewes ate no food that was mixed with the synthetic repellent, whereas nine of the 10 consumed no food laced with the total extract.

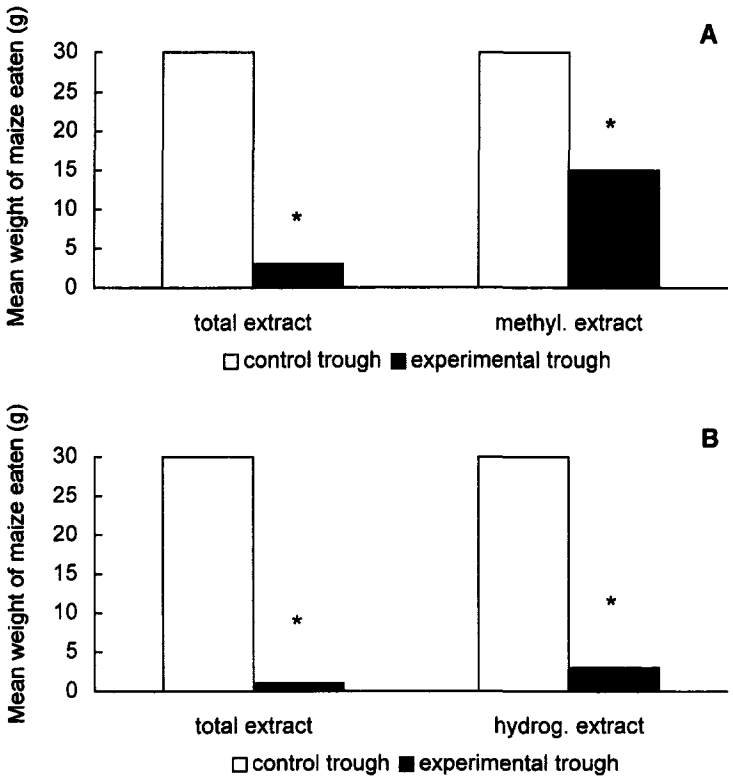

FIG. 4. Comparison of the effects of the total extracts of dog feces and the methylated (methyl., A) or the hydrogenated (hydrog., B) extracts derived from them on consumption of food by six ewes. *$P \leq 0.05$.

34	Butanoic acid	97	Non identified acid	102	Hexadecenoic acid
36	3-Methylbutanoic acid	98	Non identified acid	104	Octadecanoic acid
83	Indole	99	Pentadecanoic acid	105	Octadecenoic acid
85	Dodecanoic acid	101	Hexadecanoic acid	106	Octadecadienoic acid
95	Tetradecanoic acid				

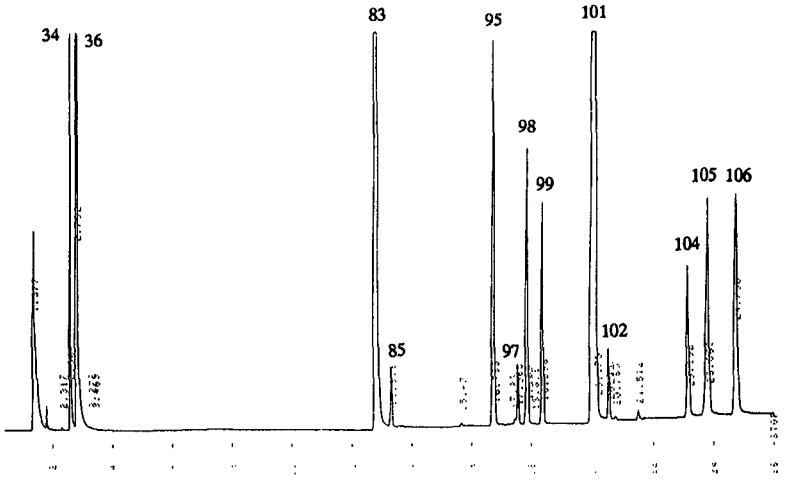

FIG. 5. Chromatogram of the synthetic mixture (condition 1, see Methods and Materials). Unidentified acids (numbers 97 and 98) were trapped by micropreparative gas chromatography from the total extract and added to the synthetic mixture.

TABLE 3. COMPARISON OF EFFECTS OF TOTAL EXTRACT OF DOG FECES AND SYNTHETIC MIXTURE DERIVED FROM IT ON CONSUMPTION OF FOOD BY 10 EWES FROM CONTROL AND EXPERIMENTAL TROUGHS, EACH CONTAINING 30 g OF MAIZE

	Mean weight of maize eaten (g ± SD)		
	Control trough	Experimental trough	P^a
Total extract	30 ± 00	01 ± 04	≤0.01
Synthetic mixture	30 ± 00	19 ± 14	≤0.05

[a]Wilcoxon test.

Repellent Effects of Single Compounds. As indole was the major component of the total extracts and has a fecal odor for humans, its repellent effect was tested. No repulsion was observed, however, because all 10 ewes tested ate all the maize from the control and experimental troughs (30 g in both cases). The same results were obtained with quinoline, another nitrogenous compound identified in the total extract.

DISCUSSION

Our results provide, for the first time, information concerning the chemical nature of the components of dog feces that function as feeding repellent in sheep. Sheep respond more to odors from potential predators (dog and wolf) than to conspecific odors. Results of behavioral and chemical studies of the odor of dog feces suggest that repulsion may be due to several compounds acting synergically. The odorous signal appears to be a cocktail of fatty acids ranging in chain length from C_4 to C_{18} (major constituents of the total extract) mixed with a smaller proportion of neutral compounds.

Results from the first experiment demonstrate that the odor of fecal matter from potential predators of sheep (dog and wolf) have a high repellent effect in this species. Similar predator effects have been observed in sheep (Pfister et al., 1990; Arnould and Signoret, 1993), goats (Weldon et al., 1993), and cattle (Pfister et al., 1990) with feces from Felidae or Canidae. In our experiment, previous experience with the odors of Canidae cannot explain the observed reaction because the sheep of the laboratory flock were kept indoors and had no previous contact with dogs or wolves for several generations. The odor of pig feces had a weak repellent effect when compared with the fecal odor of potential predators. This result strengthened our previous observations (Arnould and Signoret, 1993).

In our study, sheep were also repelled by feces from conspecifics. Weldon et al. (1993) failed to observe such an intraspecific effect in goats, but Dohi et al. (1991) have observed that cattle did not fed from a trough containing conspecific feces. Other intraspecific aversions have been described in horses and cattle living in pastures (Ödberg and Francis-Smith, 1976; Marnane et al., 1982). In our experiments, comparison of the repellent efficiency of fecal matter from predators and conspecifics indicates that the effectiveness of predator odors is higher than that of conspecifics. Some nonspecific chemical compounds may be common to fecal matter from different species, but in different proportions and/or different amounts. These variations could therefore explain the differential effectiveness observed between sheep, pig, or dog/wolf feces (conspecific, nonpredator, or predator species). It is also likely that the odor of feces is related to the diet of the animals.

The odor of only a 50-μl fraction of the 2-ml pentane extracts obtained from 200–300 g of feces (feces from pig, dog, and wolf) had an effect similar to that of 35 g of the corresponding crude feces. Thus, these extracts seem even more potent than the feces themselves. Similar observations have been reported before. According to Vernet-Maury (1980), an extract of fox feces, as well as one compound isolated from it (2,5-dihydro-2,4,5-trimethylthiazoline), induced fright responses in laboratory rats that were higher than those elicited by the odor of fox feces. In our study, a chemical analysis of the compounds active as repellent was possible because the activity of the feces was completely maintained in the pentane extracts.

Slight variations were observed in the chromatographic profiles of the volatile compounds of a series of extracts of dog feces (samples of feces were obtained from a single animal always fed with the same food). While the profiles were of a similar shape, the absolute peak heights did vary (approximately two- to three-fold) from one extract to another. These variations may explain fluctuations in the activity of the total extracts between tests. Similar variations have been described in the constituents of the anal gland secretions from foxes, coyotes, and dogs (Albone and Fox, 1971; Preti et al., 1976).

The quantitatively dominant components extracted from dog feces are fatty acids and indole. These classes of components have also been identified in excretions and secretions of numerous mammalian species as well as in putrefied products. Fatty acids have been identified in lion and rabbit feces (Goodrich et al., 1981; Abbott et al., 1990), in anal gland secretions from Canidae (Albone and Fox, 1971; Preti et al., 1976; Albone and Grönneberg, 1977; Raymer et al., 1985; Buglass et al., 1990) and Mustelidae (Sokolov et al., 1980; Brinck et al., 1983; Jacob and Schliemann, 1983, 1986; Davies et al., 1988), and also in putrefied eggs (Bullard et al., 1978). Indole has been identified in lion feces (Abbott et al., 1990) and in the anal gland secretions of Canidae (Raymer et al., 1985) and Mustelidae (Crump, 1980a, b; Sokolov et al., 1980; Brinck et al., 1983). Conversely, in our study only one sulfur-containing component (2-propylthiophene) has been identified, whereas this class of components occurs commonly in excretion and secretion products from carnivores. Sulfur-containing compounds could be present in concentrations too small for mass spectral identification. Furthermore, some of the quantitatively major components of the total extract could mask components present in smaller amounts.

The synthetic mixture tested had a lower repellent effect than the total extract. Fatty acids are probably involved in the observed repulsion, as feeding behavior of sheep was not modified in tests performed with indole. Tiedman et al. (1976) have shown that the lipidic fraction from a product derived from putrescent fish and eggs is repellent to deer. However, the intensity of the repulsion was not specified. In our previous experiments (Arnould and Signoret,

1993), we have reported that sheep were only partially repelled in tests performed with the Big Game Repellent (M.G.K., Minneapolis, Minnesota), a product made from putrescent whole-egg solids. Responses obtained in our experiment with the synthetic mixture are consistent with these results. The fatty acids, or at least some of them, seem to be involved in the repellent effect of dog feces, but they have only a partial effect and some quantitatively minor compounds such as aldehydes, alcohols, and methyl-ketones may also contribute to this effect. Both acidic and neutral components appear to be essential for the formation of the repellent signal of the odor of dog feces. Goodrich (1983) has proposed a similar hypothesis concerning territorial messages from the odor of rabbit anal glands. It is interesting to note that Vernet-Maury (1980) observed that the fright-inducing effect of fox feces for laboratory rats is mimicked by one compound alone (2,5-dihydro-2,4,5-trimethylthiazoline), whereas our results indicate that the effect of dog feces is due to several compounds acting synergically.

The carboxylic groups are directly involved in the repulsion effect because methylation has a tendency to reduce repellency. Double bonds do not appear to be involved in the activity of the total extract of dog feces since hydrogenation has no effect on repellent activity. These results differ from those obtained by Tiedman et al. (1976), who concluded that repellency of a product derived from putrescent eggs and fish was dependent on the presence of double bonds in the lipid material because hydrogenation suppressed all repellency.

Indole has a strong fecal odor, but has no repellent activity. This might be explained by the fact that this compound is not specific: it is observed in numerous biological products from many different species and origins (Albone, 1984).

2,5-Dihydro-2,4,5-trimethylthiazoline, a single compound isolated from fox feces, has not been identified in dog feces. This compound, which reduces vole attacks in orchard blocks (Sullivan et al., 1988), has no repellent effect on sheep (Arnould et al., 1994). These results underline the fact that a product avoided by one species may be ineffective for another one. 2,5-Dihydro-2,4,5-trimethylthiazoline may have an effect only on rodents (Arnould et al., 1994).

Individual food choice tests used in these experiments allow reliable measures of repellency and are suitable for identifying specific volatile signal involved in the food repellent effect of dog feces. Identification of this signal could be facilitated by the use of several methods. For example, volatile constituents of dog and wolf feces could be compared, as these two species are closely related. Identification of the major constituents of an extract from wolf feces has been initiated. Fatty acid concentrations appeared to be lower in this extract than in an extract from dog feces (unpublished results), but both are similarly effective. Furthermore, extraction of the volatiles from dog feces by the headspace technique could also facilitate identification. Volatiles trapped by this method are

reduced to those effectively emitted by feces. Conversely, in our study, the volatile compounds were trapped by simultaneous steam distillation–extraction (a method previously used by E. Vernet-Maury, personal communication). In this method, fecal matter is heated and some volatile compounds may have been partly modified. Comparison of the components common to the extracts obtained by these two methods could provide information on a new series of compounds that could be tested on animals.

This study is a first step in the identification of the chemical constituents that may be involved in the formation of the repellent signal (for ungulates) contained in the odor of dog feces. It shows clearly the complexity of the odorous signals by which information of behavioral importance is communicated. The use of reliable behavioral tests and several biochemical methods for volatile analysis could contribute to the development of synthetic repellents from natural active compounds.

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QUANTIFYING IN SITU RATES OF PHLOROTANNIN SYNTHESIS AND POLYMERIZATION IN MARINE BROWN ALGAE

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Abstract—Using three species of marine brown algae, we describe a stable isotope labeling technique to quantify: (1) in situ rates of phlorotannin synthesis, (2) phlorotannin polymerization or aging, and (3) the related allocation of carbon resources to secondary metabolism. In our field and laboratory assays, *Lobophora variegata* (Bahamas), *Sargassum pteropleuron* (Bahamas), and *Fucus distichus* (California, USA) assimilated various quantities of ^{13}C, but all allocated $\leq 1\%$ of the assimilated carbon to the production of phlorotannins. We quantified rates of phlorotannin synthesis both as micrograms of compound produced per gram of tissue per unit of time and as micrograms of compound produced per gram of C assimilated per unit of time. Rates of synthesis, normalized to account for differences in potential photosynthetic rates, are comparable to previously reported rates of phlorotannin accumulation. The aging of phlorotannins from low- (<30 kDa) to high- (>30 kDa) molecular-size polymers was observed in *S. pteropleuron* within a 28-hr period. Our results indicate that, using this labeling technique, it is possible to make precise measurements of allelochemical metabolism and resource allocation, which are useful both in critically evaluating the assumptions made by ecological models of plant chemistry and in estimating the partial metabolic cost of specific secondary metabolites.

Key Words—Phlorotannins, allelochemicals, biosynthesis, turnover, cost vs. benefit, resource allocation, brown algae, metabolism.

INTRODUCTION

Within the Phaeophyta, polyphenolic secondary metabolites occur at ecologically significant concentrations throughout the orders Ectocarpales, Dictyotales,

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Laminariales, and Fucales. These brown algal phlorotannins are acetate-malonate-derived polymers of phloroglucinol (1,3,5-trihydroxybenzene), which have been implicated as herbivore deterrents, digestion reducers, and antibacterial agents and thus are putative defensive agents (see Rosenthal and Jansen, 1979; Norris and Fenical, 1982; Ragan and Glombitza, 1986; Hay and Fenical, 1988; Bernays et al., 1989; Steinberg, 1992a,b; Targett et al., 1995 for reviews). In plant–animal interactions where phlorotannins are influential, their bioactivity is both concentration dependent and also a function of their molecular size profile (Boettcher and Targett, 1993). Total phlorotannin concentrations >5% dry mass occur throughout the temperate and tropical Atlantic and temperate Pacific marine environments (Ragan and Glombitza, 1986; Steinberg, 1992a; Targett et al., 1992, 1995 for reviews). Within a given area, inter- and intraspecific phlorotannin concentrations have been shown to vary as a function of salinity (Ragan and Glombitza, 1986), nutrient availability (Ilvessalo and Tuomi, 1989; Yates and Peckol, 1993; Arnold et al., 1995; Peckol et al., 1996), herbivore intensity (Van Alstyne, 1988; Arnold and Targett, unpublished), irradiance levels (Ragan and Glombitza, 1986; Arnold and Targett, unpublished), season (Ragan and Jensen, 1978; Geiselman, 1980; Ragan and Glombitza, 1986), plant size (Denton et al., 1990), age (Pederson, 1984), and tissue type (Steinberg, 1984; Tugwell and Branch, 1989; Tuomi et al., 1989). Investigations regarding the ecological roles of polyphenolic secondary metabolites, including their effects on herbivores and their patterns of inter- and intraspecific variation, have been influential in shaping current ecological theories. Ecological theories such as plant apparency and optimal defense (Feeny, 1976; Rhoades and Cates, 1976), resource availability (Coley et al., 1985; Bazzaz et al., 1987; Bryant et al., 1987), and growth–differentiation balance (reviewed by Herms and Mattson, 1992; Lerdau et al., 1994; Tuomi, 1992) attempted to account for the inter- and intraspecific patterns of allelochemistry by balancing putative improvements in plant fitness, as conveyed by secondary metabolites, with the costs implied by metabolite synthesis and maintenance. In both marine and terrestrial systems, experimental tests of these theories often yield equivocal results, and it has been suggested that the assumptions underlying these theories may not apply equally to all classes of secondary metabolites (e.g., see Reichardt, 1991 and references therein). We suggest that our understanding of the biotic and abiotic factors that control the production of secondary metabolites is limited because experiments to date have focused primarily on correlations between plant stresses (nutrient limitation, irradiance levels, herbivory) and static metabolite concentrations, rather than the underlying rates of metabolite synthesis, turnover (i.e., degradation, remetabolism), and exudation.

Most terrestrial studies investigating cost–benefit relationships (see Coley, 1986; Briggs and Shultz, 1990; Han and Lincoln, 1994 for examples) and all those involving marine systems have focused on correlations between static

concentrations of defensive chemicals and related changes in plant growth or reproductive efforts. For example, Steinberg (1995) found a correlation of decreased growth and increased phlorotannin content for the brown alga *Ecklonia radiata* in the spring, but not the autumn. Yates and Peckol (1993) demonstrated a similar correlation between plant growth and phlorotannin concentrations in the rockweed *Fucus vesiculosus* at nutrient-enriched sites, but not unenriched sites. Pfister (1992) found that frond phlorotannin levels in the kelp *Alaria nana* were correlated with reduced growth, but that sporophyll phlorotannin levels were correlated with increased growth and plant size. These studies focused on factors that ultimately affect plant fitness and thus had direct ecological consequences. However, such correlations do not necessarily indicate causation. In addition, because of the static nature of the measurements of phlorotannin concentrations, this approach does not provide much information regarding the synthesis and/or turnover of allelochemicals. Only a few studies have taken a metabolic approach, which focuses on plant metabolism and defines cost in terms of resources allocated to the production and maintenance of secondary metabolites. Chew and Rodman (1979) used the metabolic approach in estimating the cost of several nonphenolic, nitrogen-containing compounds, such as cyanogenic glycosides, glycosinolates, and nonprotein amino acids, in vascular plants. They calculated cost in terms of substrate and enzyme requirements of known secondary pathways (also see Lerdau and Gershenzon, 1997). Gershenzon et al. (1993) and Mihaliak et al. (1991) utilized radioactive precursors to demonstrate that the rate of terpene turnover in several species of rooted plants was low and suggested that the paradigm of terpenes as short-lived allelochemicals (Burbott and Loomis, 1969; Croteau et al., 1972) is flawed, although they made no direct attempt to estimate cost (Gershenzon, personal communication).

Here we describe a technique for measuring both in situ rates of phlorotannin synthesis and polymerization as well as the allocation of carbon resources to secondary metabolism in species of marine brown algae from the orders Fucales and Dictyotales. In both laboratory and field assays, macrophyte assimilation of a stable isotope substrate, [^{13}C] $NaHCO_3$ (in the form of dissolved inorganic carbon in seawater), and the subsequent incorporation of the label into plant tissues was used to quantify phlorotannin synthesis and, for *S. pteropleuron*, polymerization. The proportional allocation of assimilation carbon to the production of algal phlorotannins was also determined in an attempt to estimate the partial metabolic cost of polyphenolic compounds under these conditions.

METHODS AND MATERIALS

Preparation of ^{13}C-Labeled Seawater. For all assays, filtered seawater was acidified to pH 3 by addition of HCl (1 N) and the dissolved inorganic carbon

(DIC), present entirely as dissolved carbon dioxide, was removed by rapidly bubbling CO_2-free air into solution for 1 hr. The absence of total inorganic carbon was confirmed using a nondispersive infrared analyzer according to the methods of Sharp (1973). Prior to the addition of [^{13}C] $NaHCO_3$ (Europa Scientific, 99% pure), the pH was adjusted to pH 6 by addition of NaOH (1 N). The ^{13}C-labeled sodium bicarbonate was added to achieve a concentration of 2.24 mM [^{13}C] DIC and the pH was readjusted to 7.9–8.0.

Field Experiments. Lobophora variegata (Lamouroux) Womersley and *Sargassum pteroplueron* Grunow were collected by scuba divers from the waters surrounding Pigeon Cay, Andros Island, Bahamas (24°52′N, 77°53′W) in March of 1996. Healthy, epiphyte-free individuals were carefully removed from the substrata and brought to the surface in shaded containers. *L. variegata* (decumbant form) was collected from a backreef habitat (17 m) where it was the dominant algal species covering hard substrata. Twenty-four individuals of *L. variegata* were placed in a single 4-liter polycarbonate incubation chamber containing labeled seawater. *Sargassum pteroplueron* was collected from a shallow (1 m) sandflat and 12 individuals of *S. pteroplueron* were placed in a separate 4-liter polycarbonate incubation chamber. The ratio of seaweed wet mass (WM) per incubation volume was maintained constant at ca. 40g WM/liter to facilitate comparisons between experiments. The incubation chambers containing seaweeds were immediately (within 60 sec) returned to depth at the sites of collection and secured 0.5 m above the bottom for 3.5 hr. In situ irradiance levels and seawater temperatures were measured at each site during the midday incubation periods. Following the incubation period, plants were removed from the chambers, rinsed to remove unincorporated label, and held at depth in perforated, herbivore-excluding polycarbonate containers. For both *S. pteroplueron* and *L. variegata*, plants ($N \geq 3$) were sampled from their respective habitats at the following intervals: immediately following incubation (3.5 hr), and at 24, 48, and (conditions permitting) 72 hr. Specimens ($N \geq 3$) collected and sampled prior to the incubations were used to determine background concentrations and isotope ratios of algal phlorotannins. All plant tissue samples were immediately frozen in liquid nitrogen and transported to the University of Delaware for analysis.

Laboratory Experiments. Fucus distichus Linnaeus was collected from Point Arguello, California, in April 1996. Plants ranged in length from 10 to 20 cm and were largely epiphyte-free. At the University of Delaware, 15 individuals were allocated to a 180-liter recirculating seawater system containing 0.2 μm filtered seawater. The following conditions were maintained: 100 μmol photons/m^2/sec irradiance, 12L:12D cycle, 17–19°C, 100 μmol/N/liter and a flow rate of 10 liters/min. A fraction (105 liters) of the seawater was renewed every 24 hr. Following a 48-hr acclimation period, *F. distichus* (19 g WM/liter) was

distributed to a polycarbonate incubation chamber containing labeled seawater. The chamber was floated directly in the recirculating system and stirred intermittently throughout the 6-hr incubation period. Following incubation, plants were removed from the chamber, rinsed to remove unincorporated label, and returned to the recirculating system. Individual plants ($N = 3$) were sampled prior to incubation (background), immediately following incubation ($t = 6$ hr), and at 24, 72, 144, and 192 hr. All tissue samples were blotted dry and stored at −80°C.

Isolation of Phlorotannins. Whole plants (≥5 g WM) from each sampling interval ($N = 3$/interval) were blotted dry, weighed, and homogenized in liquid nitrogen. Subsamples of homogenized individuals were used in the determination of plant carbon–nitrogen ratios (Carlo Erba NA1500 elemental analyzer) and the calculation of ^{13}C assimilation rates. The remaining tissue from each individual was extracted individually, in 25 ml 70% MeOH (aq)/g tissue WM for 24 hr under a nitrogen atmosphere and in the dark. Extracted phlorotannins were purified by a method adapted from Ragan and Glombitza (1986). Extraction solutions were filtered to remove particulate material, concentrated in vacuo, and partitioned against chloroform (all solvents were Burdick and Jackson, HPLC grade). Subsequently, aqueous fractions were repeatedly partitioned against equal volumes of ethyl acetate. The ethyl acetate fractions, which contain most, but not all, of the extracted polyphenolics, were collected, pooled, and concentrated. Pure phlorotannins were then obtained by repeated vacuum liquid chromatography (VLC) on dry cellulose particles (Sigmacell T-101, Sigma Corp.) using a solvent gradient of increasing polarity, from 9:1 chloroform–acetone to 5:2 acetone–methanol. Since the purity of isolated phlorotannins is of utmost importance, samples were analyzed by a variety of qualitative and quantitative methods, including thin-layer chromatography (TLC), elemental analysis (CHN composition), and proton nuclear magnetic resonance (^1H NMR) of acetylated derivatives according to the methods modified from Ragan and Glombitza (1986). For phlorotannins isolated from *S. pteropleuron*, replicate whole plant samples ($N = 3$) were pooled and the compounds were separated into nominal molecular size fractions (<30 kDa and >30 kDa) using double-layers of Amicon stirred membrane filters (Boettcher and Targett, 1993).

The total phenolic content of homogenized tissues from plants sampled at each time interval ($N = 3$/interval) was determined using a modified Folin-Denis assay, which uses reduced sample sizes (Hatch et al., 1993; Arnold et al., 1995). Phloroglucinol equivalents were calculated by comparison with a standard curve of desiccated phloroglucinol (Sigma Corp.) diluted to 0–20 μg/ml in the extraction solution. Wet–dry mass ratios were determined by homogenized plants of each species by drying replicate samples at 60°C for 48 hr. Estimated phlorotannin concentrations are reported as a percent of tissue dry

mass. The percent carbon and nitrogen concentrations of homogenized whole plant tissues were determined in conjunction with isotope analyses.

Isotopic Analyses. Analyses of the $^{13}C/^{12}C$ ratios from plant tissue and isolated phlorotannin samples (SIRA Series II isotope ratio mass spectrometer, Duke University Phytotron) yield $\delta^{13}C$ values as: $\delta^{13}C = R_{sample}/R_{standard}$, where $R = {}^{13}C/{}^{12}C$ (the molar abundance ratio). Internal standards are calibrated to Pee Dee belemnite (Oztech), a carbonate mineral standard, as reviewed in Boutton (1991). Homogenized tissue samples were analyzed to determine algal assimilation of ^{13}C during the incubation by conversion of $\delta^{13}C$ values to milligrams of ^{13}C incorporated per gram of plant dry matter per day. In calculating these values, the natural (background) ^{13}C content of algal tissues, as measured prior to incubation, was subtracted from values of ^{13}C incorporation. Phlorotannin synthesis was calculated for each postincubation sample as:

$$(1)\ \frac{\text{mg } {}^{13}\text{C in PPS}}{\text{mg PPS}} \times \frac{100}{\text{\% PPS that is C}} = \frac{\text{mg PPS composed entirely of } {}^{13}\text{C}}{\text{mg PPS}}$$

$$(2)\ \frac{\text{mg PPS composed entirely of } {}^{13}\text{C}}{\text{mg PPS}} \times \frac{\text{\% phlorotannins in plant (DM)}}{0.1} = \frac{\text{mg } {}^{13}\text{C phlorotannins}}{\text{g plant tissue (DM)}}$$

$$(3)\ \frac{\text{mg } {}^{13}\text{C phlorotannins}}{\text{g plant tissue (DM)}} - \frac{\text{mg } {}^{13}\text{C phlorotannins (background)}}{\text{g plant tissue (DM)}} = \text{net synthesis (mg/g DM)}$$

where PPS is plant phlorotannin sample, that is isolated phlorotannins that were analyzed by mass spectrometry. Equation 1 calculates the fraction of phlorotannins containing ^{13}C in the total plant phlorotannin sample. In essence this calculation reshuffles the available carbon isotopes to assemble two groups of phlorotannin compounds—those composed of ^{13}C and those composed of ^{12}C. In actuality, phlorotannin molecules would be composed of both isotopes in various proportions. Equation 2 expresses the fraction calculated in equation 1 in terms of phlorotannins per gram of plant tissue, as determined by the Folin-Denis assay for individual plants. It is conventional to express phlorotannin concentrations as the percentage of dry mass; therefore, it is necessary to divide by 0.1 to transform the value to milligrams of phlorotannin per gram of plant dry mass. The net abundance of new phlorotannins relative to background samples is calculated by equation 3. When integrated over time, this allows synthesis

rates to be expressed as milligrams of phlorotannins per gram of plant dry mass per day. Turnover rates, also described as milligrams of phlorotannins per gram of plant dry mass per day, would represent negative net synthesis and would describe the rate of loss of label—rather than its accumulation—from the phlorotannin pool, although by themselves they would give no indication of how the loss occurs. Rates of synthesis and polymerization are presented as both milligrams of phlorotannins per gram of plant dry mass per hour and milligrams of phlorotannins per gram of carbon assimilated per hour. The isotopic analyses of homogenized plant tissue subsamples (described in the previous section) provides measures of grams of carbon assimilated per gram of plant dry mass allowing for the relative ^{13}C enrichment of the isolated phlorotannins to be calculated. These data are useful for comparative purposes because they are calculated independently of Folin-Denis measures of phlorotannin pool-size.

RESULTS

Isolated phlorotannins developed with 5:2 acetone–methanol on silica-gel TLC plates (Merck) stained positive for polyphenolics with a sulfuric acid–vanillin spray solution. When examined by ^{1}H NMR (JEOL FX90Q), the acetylated derivatives exhibited characteristic signals between 1.6 and 2.3 ppm (acetyl groups) and between 6.2 and 7.1 ppm (aromatic hydrogens). Signals between 2.9 and 6.2 ppm, which indicate the presence of aliphatic contaminants (K. W. Glombitza, personal communication), were absent, as were signals between zero and 1.6 ppm. For *L. variegata*, *S. pteropleuron*, and *F. distichus*, our methods provided 0.5–1 mg phlorotannins/g plant tissue WM extracted. The percent carbon and nitrogen composition of isolated phlorotannins was within the range reported by Ragan and Glombitza (1986). For the three species reported here and all others surveyed to date, the $\delta^{13}C$ values recorded for unenriched phlorotannins were identical to the $\delta^{13}C$ values of unenriched plant tissues, i.e., there was no discrimination between ^{12}C and ^{13}C during secondary metabolism (data not shown).

Lobophora variegata assimilated 462 ± 39.91 μg ^{13}C/g tissue DM during the 3.5-hr incubation at an average irradiance of 158 μmol photons/m^2/sec (Table 1). Results of tissue analyses are also summarized in Table 1. Phlorotannin concentrations ranged from 4.89 ± 0.83 to 2.87 ± 0.45%DM (Table 2). During the total 50-hr assay period, *L. variegata* produced a total of 4.39 ± 1.48 μg phlorotannins/g tissue DM (Figure 1). During the initial 3.5-hr incubation period, a mean of 3.12 μg phlorotannins/g tissue DM, or 71.1% of the total, were produced. The remaining 28.9% were produced during the postincubation period from ^{13}C, which had been assimilated by the plant but not immediately utilized for phlorotannin synthesis. In *L. variegata*, 2.90 μg

TABLE 1. RESULTS OF BIOCHEMICAL ANALYSES AND CALCULATIONS OF PHLOROTANNIN BIOSYNTHESIS[a]

	Field experiments		Lab experiments
	L. variegata	*S. pteropleuron*	*F. distichus*
Habitat			
Location	Bahamas	Bahamas	California[d]
Depth (m)	17	1–2	Intertidal
Irradiance (μmol photons/m^2/sec)	158	1600	100
Plant tissues			
Nitrogen (%)	0.83 ± 0.03(3)	1.13 ± 0.22(3)	1.81 ± 0.19(4)
C:N ratio	36.66 ± 2.17(3)	18.71 ± 1.65(3)	19.53 ± 1.34(4)
Phlorotannins (% DM)[b]	3.55 ± 0.36(24)	4.32 ± 0.14(12)	4.75 ± 0.29(18)
δ^{13}C value[c]	−28.24 ± 0.49(3)	−26.27 ± 1.05(3)	−28.94 ± 0.02(3)
Assimilation			
Incubation time (hr)	3.35	3.35	6.00
C assimilated (μg/g)	462 ± 39.91(3)	2082 ± 668.55(3)	292 ± 14.44(3)
Biosynthesis			
Carbon allocated (%)	0.88(3)	0.29(3)	1.00(3)
Product (μg/g/hr)	4.39 ± 1.48(3)	46.00 ± 22.2(3)	5.32 ± 0.98(3)

[a]Errors indicated are ± 1 SE. Sample sizes are indicated within parentheses.
[b]Mean phlorotannin concentration of all plants.
[c]Original isotopic composition of collected plants.
[d]Laboratory experiments conducted at the University of Delaware.

TABLE 2. MEANS, STANDARD ERRORS, AND TUKEY HSD PAIRWISE COMPARISONS OF WHOLE-PLANT PHLOROTANNIN CONCENTRATIONS (% DM) AT SPECIFIC TIME INTERVALS FOR *Lobophora variegata, Sargassum pteroplueron*, AND *Fucus distichus*[a]

	Sampling interval						
	BG	Post	1 day	2 days	3 days	6 days	8 days
Lobophora variegata							
Data:							
% DM	4.89	3.50	2.87	2.93			
N	6	6	6	6			
SE	0.83	0.60	0.45	0.79			
Comparisons							
BG	1.000						
Post	0.496	1.000					
1 day	0.193	0.914	1.000				
2 days	0.212	0.932	1.000	1.000			
Sargassum pteroplueron							
Data							
% DM	4.04	4.59	4.80	3.89			
N	3	3	3	3			
SE	0.12	0.34	0.08	0.05			
Comparisons							
BG	1.000						
Post	0.246	1.000					
1 day	0.083	0.855	1.000				
2 days	0.936	0.112	0.037*	1.000			
Fucus distichus							
Data							
% DM	4.58	4.25	5.26		5.56	3.62	4.72
N	6	6	3		3	3	3
SE	0.46	0.65	0.49		0.11	0.51	0.29
Comparisons							
BG	1.000						
Post	0.995	1.000					
1 day	0.951	0.793	1.000				
3 days	0.812	0.575	0.999		1.000		
6 days	0.824	0.964	0.491		0.318	1.000	
8 days	1.000	0.990	0.991		0.937	0.826	1.000

[a]Sampling intervals include background (BG) and postincubation (Post) periods. Tukey HSD pairwise comparisons are the probability of each pair being significantly different. Those significant at $\alpha = 0.05$ are indicated by an asterisk.

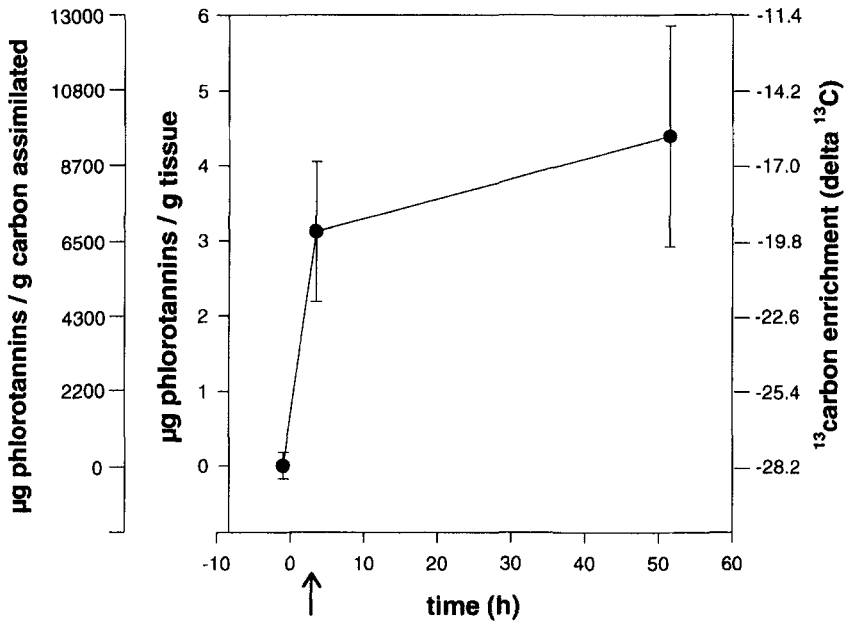

FIG. 1. *Lobophora variegata* (Bahamas). In situ biosynthesis of phlorotannins. Time "zero" represents background levels of ^{13}C in phlorotannins and is followed by the start of the 3.5-hr incubation period (arrow). Biosynthesis, expressed as the mean of three replicates ± 1 SE, is reported as µg phlorotannins/g tissue, µg phlorotannins/g ^{13}C assimilated, and as the ^{13}C enrichment of the total phlorotannin pool.

^{13}C/g tissue, or 0.63% of the total assimilated carbon, was allocated to phlorotannin production during incubation. In all, 4.08 µg ^{13}C/g tissue, or 0.88% of the assimilated ^{13}C, was allocated for phlorotannin synthesis during the 50-hr assay period.

Sargassum pteropleuron assimilated 2082 ± 668.55 µg ^{13}C/g tissue DM during the incubation at an average irradiance of 1600 µmol photons/m^2/sec (Table 1). Whole-plant phlorotannin concentrations ranged from 4.80 ± 0.08 to 3.89 ± 0.06%DM (Table 2). During the initial incubation period, *S. pteropleuron* produced 46.0 ± 22.2 µg phlorotannins/g tissue (Figure 2). The average value of 38.4 µg phlorotannin/g tissue maintained during the postincubation period was similar to that already produced during the incubation. Of the total 2082 µg ^{13}C/g tissue DM assimilated during incubation, 6.02 µg ^{13}C/g tissue, or 0.29%, was allocated to phlorotannin production during the incubation period. There was no significant phlorotannin production, above that described for the incubation period, observed during the postincubation period. Low-molecular-

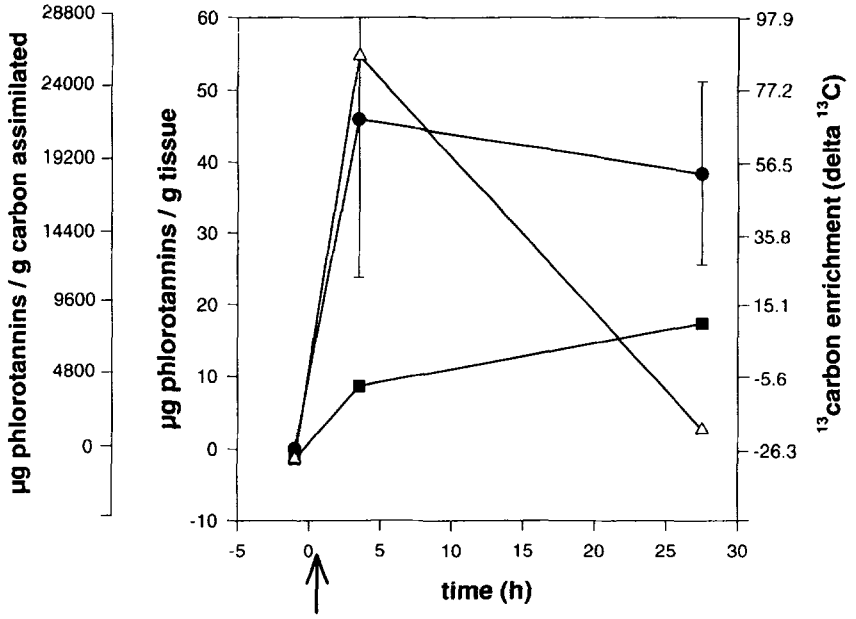

FIG. 2. *Sargassum pteropleuron* (Bahamas). In situ biosynthesis of phlorotannins. Time "zero" represents background levels of ^{13}C in phlorotannins and is followed by the start of the 3.5-hr incubation period (arrow). Total biosynthesis, expressed as the mean of three replicates ± 1 SE (●), is reported as µg phlorotannins/g tissue, µg phlorotannins/g ^{13}C assimilated, and as the ^{13}C enrichment of the total phlorotannin pool. The biosynthesis of <30 kDa (▲) and >30 kDa (■) phlorotannin fractions in *S. pteropleuron* demonstrates the aging or polymerization of phloroglucinol units over time.

size phlorotannins (≤30 kDa) initially incorporated ^{13}C to a greater extent than did high-molecular-size phlorotannins (≥30 kDa), although the trend was reversed by the end of the 28-hr assay (Figure 2).

Fucus distichus assimilated 292 ± 14.44 µg of ^{13}C/g tissue DM during the 6-hr incubation at an average irradiance of 110 µmol photons/m^2/sec (Table 1). Phlorotannin concentrations of macrophytes ranged from 5.56 ± 0.12 to 3.62 ± 0.51%DM (Table 2). During the entire eight-day assay period, *F. distichus* produced a maximum of 5.32 ± 0.98 µg phlorotannins/g tissue (Figure 3). During the incubation period, macrophytes produced 2.84 µg phlorotannins/g tissue DM, or 53.4% of the total. The remaining 46.6% was produced during the postincubation period from ^{13}C that had been assimilated but not immediately used for phlorotannin synthesis. Of the overall 292 ± 14.44 µg ^{13}C/g tissue DM assimilated by *F. distichus* during incubation, 1.56 µg ^{13}C/g tissue, or 0.53% of the total, was allocated to phlorotannin production during the incu-

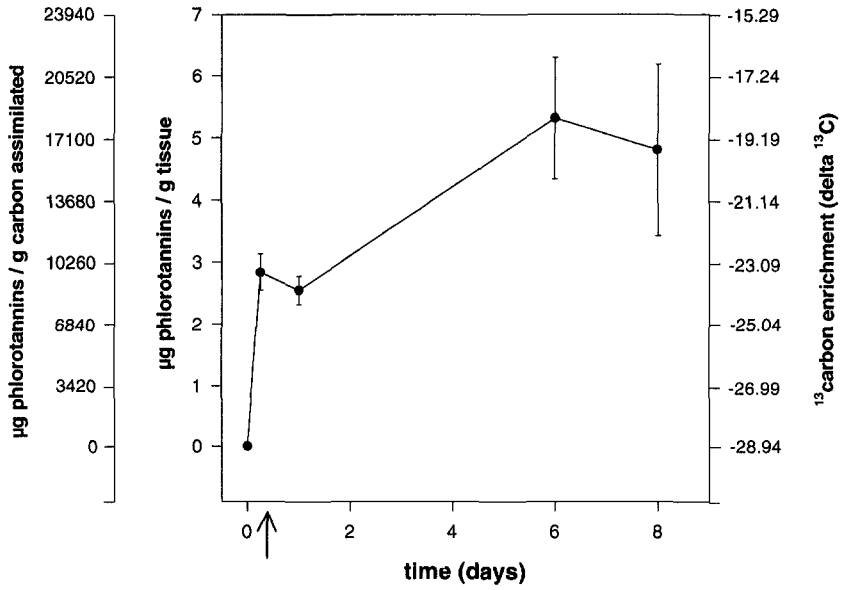

FIG. 3. *Fucus distichus* (California, USA) Biosynthesis of phlorotannins by *F. distichus* acclimated to laboratory conditions. Time "zero" represents background levels of ^{13}C in phlorotannins and is followed by the start of the 6-hr incubation period (arrow). Biosynthesis, expressed as the mean of three replicates ± 1 SE, is reported as µg phlorotannins/g tissue, µg phlorotannins/g ^{13}C assimilated, and as the ^{13}C enrichment of the total phlorotannin pool.

bation period. On the other hand, 1.36 µg ^{13}C/g tissue, or 0.47% of the total carbon resources, was allocated to phlorotannin production during the eight-day postincubation period. In all, 2.92 µg ^{13}C/g tissue, or 1.00% of the assimilated ^{13}C, was allocated for phlorotannin synthesis.

DISCUSSION

The assimilation of ^{13}C, as DIC, and its subsequent incorporation into secondary metabolism provides a means of quantitatively measuring in situ rates of phlorotannin synthesis and polymerization. The technique offers an alternative to radiolabeling methods commonly employed and, although it was utilized here to examine phlorotannins, it should be equally applicable to other carbon-based secondary metabolites. It is particularly useful in field settings where radioisotopes may be prohibited. Stable isotopes have also been shown to be useful in examining the primary metabolism of marine plants (Wu and Gretz, 1993). In

our study, the rates of carbon assimilation varied by species, from 48.7 (*F. distichus*) to 581.1 (*S. pteropleuron*) μg C assimilated/g/hr. These rates are considerably lower than the potential photosynthetic rates described for similar species (see Littler and Arnold, 1984), in part because plants were incubated without constant stirring. To account for differences in photosynthetic carbon assimilation, measurements of phlorotannin synthesis were normalized to milligrams of compound produced per gram of carbon assimilated for each time interval. While rates of assimilation varied among the species studied, no species allocated more than ca. 1% of the assimilated carbon to the production of phlorotannins themselves, although additional resources may have been allocated to the maintenance of pathways or storage structures.

Conceptually, synthesis can be described either as direct, where assimilated carbon is utilized immediately in phlorotannin metabolism, or indirect, in which the resource cycles through some other metabolic pathway or storage compound prior to being used in the production of phlorotannins. In these assays, biosynthesis measured during the incubation period may be both direct and indirect, since ^{13}C may have been immediately allocated to phlorotannin production or, in theory, reclaimed from ^{13}C previously utilized elsewhere in the plant. Synthesis measured during the postincubation period is entirely indirect, i.e., the ^{13}C source is absent. *F. distichus* clearly demonstrated significant indirect synthesis of phlorotannins, supporting the contention that these phenolic compounds may represent a net carbon sink within brown algae. It is unknown whether or not this indirect synthesis of phlorotannins from recycled acetyl CoA reserves (via the acetate–malonate pathway) is actively controlled by the plant.

In preliminary assays utilizing the tropical and temperate species described here as well as an intertidal population of *Fucus vesiculosus* (Maine, USA) the allocation of carbon resources to phlorotannin synthesis remained relatively constant for each species, regardless of changing rates of carbon assimilation. Clearly, further studies are needed in order to describe the relationship between photosynthetic carbon assimilation and resource allocation; however, it is useful to compare the rates of phlorotannin synthesis reported here to previously reported rates of phlorotannin accumulation in order to evaluate the method. At common rates of photosynthesis for morphologically similar species (Littler and Arnold, 1984) and assuming a 1% allocation of carbon to phlorotannin production, *L. variegata* could potentially produce ca. 330 μg of phlorotannin/g/day and completely replace its 40 mg/g allelochemical pool in 120 days (assuming a constant photosynthetic rate for 12 hr/day). Similarly, *S. pteropleuron* would also produce ca. 330 μg phlorotannin/g/day with a complete turnover time of 120 days, while *F. distichus* would produce ca. 175 μg phlorotannin/g/day with a complete turnover time of 260 days (Table 3). By way of comparison, Steinberg (1995) reported significant increases in the phlorotannin content of *Ecklonia radiata*, of approximately 20 mg/g, during four-week periods in both spring and

autumn. This is equivalent to a rate of 700 μg phlorotannin/g/day and would cause a plant containing 80 mg phlorotannin/g tissue to completely replace its allelochemical pool in ca. 110 days (Table 3). Arnold et al. (1995) observed, for cultured clones of *L. variegata*, an increase of 13.8 mg phlorotannin/g in 21 days in response to a reduction in nitrogen availability. This corresponds to a 660 μg/g/day rate of accumulation and a 45-day period for the complete replacement of a 30 mg/g allelochemical pool. Yates and Peckol (1993) reported changes in the phlorotannin content of *Fucus vesiculosus* from New England. At a low-nitrogen site they show an increase in phenolic content from approximately 10 mg/g in July to approximately 25 mg/g in August, which corresponds to a rate of 500 μg/g/day and would require a 40-day period for the complete turnover of polyphenolics in a plant with a concentration of 2% DM. These rates of accumulation reported in previous studies are some of the highest rates reported for whole plants and are somewhat higher than the in situ rates of synthesis we report here (Table 3). A careful examination of the literature reveals, however, that only the highest rates of accumulation have allowed differences in phlorotannin concentration to be resolved over the experimental periods. Slower rates of accumulation, such as those quantified by the alternative method we describe here, are generally not detected by previous methods or are reported as nonsignificant. While it is interesting to compare these rates of synthesis to related studies that have documented the accumulation of phlorotannins over various time periods from days to months, we stress that the synthesis rates presented here are from a limited number of species in specific habitats and are meant primarily to demonstrate the method. Nevertheless, given the broad range of species and habitats in which the accumulation of polyphenolic compounds has been observed, the similarity in rates of accumulation is remarkable.

In macrophytes maintaining a consistent concentration of phlorotannins, it is not known whether synthesis ceases and/or is depressed or, alternatively, the rate of turnover simply matches the rate of synthesis. Rates of metabolite turnover, in addition to synthesis, may greatly influence the cost of defense. The aging of low-molecular-size phlorotannins into high-molecular-size polymers, as discussed by Ragan and Glombitza (1986), was observed for phlorotannins in *S. pteropleuron*. Here we observe that polymers ≤ 30 kDa were synthesized within 5 hr following incubation and that their concentration decreased over time as the concentration of ≥ 30 kDa polymers increased. The rate at which phlorotannins age is potentially significant to their roles as defensive agents since in plant-herbivore interactions where phlorotannins are influential, their molecular size affects their bioactivity (Boettcher and Targett, 1993). The degree of polymerization also influences the reactivity of polyphenolic compounds in common spectrophotometric assays (Ragan and Glombitza, 1986; Mole et al., 1989; Waterman and Mole, 1994; Van Alstyne, 1995).

TABLE 3. COMPARISON OF PREVIOUSLY REPORTED RATES OF PHLOROTANNIN ACCUMULATION AND IN SITU MEASUREMENTS OF PHLOROTANNIN SYNTHESIS[a]

Species	Habitat	Concentration (% DM)	Synthesis (μg/g/day)	Turnover (days)	Reference
Lobophora variegata	Shallow reef	3.55	330	120	this study
Sargassum pteropleuron	Sand flat	4.32	330	120	this study
Fucus distichus	Intertidal/Lab	4.75	175	260	this study
			Accumulation (μg/g/day)		
Fucus vesiculosus	Low intertidal	~2.5[b]	330	75	Peckol et al. (1996)
Fucus vesiculosus	High intertidal	~2.8[b]	860	32	Peckol et al. (1996)
Ecklonia radiata	Subtidal	~8[b]	700	110	Steinberg (1995)
Lobophora variegata	Culture	~3[b]	660	45	Arnold et al. (1995)
Fucus vesiculosus[c]	Subtidal	~2[b]	500	40	Yates and Peckol (1993)
Fucus vesiculosus[d]	Exposed/Intertidal	~5[b]	495	100	Ilvesallo and Tuomi (1989)
Fucus vesiculosus[d]	Loose/Intertidal	~9[b]	1140	77	Ilvesallo and Tuomi (1989)
Fucus distichus[e]	Intertidal	~10[b]	1270	70	Van Alstyne (1988)
F. vesiculosus var. *spiralis*	Intertidal	3.7[b]	877	42	Arnold and Targett (unpublished)

[a] Only studies reporting significant accumulation in phlorotannins over a specified time period are shown. Where several instances of accumulation are reported in the same study, the highest rates are reported. Rates of accumulation are calculated as the increase in concentration as μg phlorotannin/g tissue/time (days).

[b] Phlorotannin concentrations of plants prior to the reported accumulation.

[c] Yates and Peckol (1993) report several additional instances of significant phlorotannin accumulation.

[d] Phlorotannin concentrations originally presented as the ratio of phlorotannins/carbon. We have transformed their data.

[e] Phlorotannin concentrations measured for mid-thallus tissues only, thus calculations apply specifically to these tissues.

The method we describe here provides information regarding the metabolism of secondary compounds and the allocation of plant resources, which is required for the critical evaluation of many ecological models of plant chemistry. It is important to note that the overall investment in allelochemicals will be underestimated unless the cost of maintaining the requisite pathways and storage structures is also considered (Zangerl and Bazzaz, 1992). As a result, the method is most useful for comparisons of cost among species sharing a particular class of secondary metabolites whose pathways and storage structures are related. Investigations concerning these aspects of secondary metabolism, which are especially scarce in marine systems, are therefore valuable in the careful application of this and other similar techniques. The various ecological and metabolic approaches to empirically estimating the cost of a particular chemical defense and their inherent limitations are described in detail by Chew and Rodman (1979), Bazzaz et al. (1987), Fagerstrom (1989), and Zangerl and Bazzaz (1992). By applying the methods of Chew and Rodman (1979) and Lerdau and Gershenzon (1997), the cost of maintaining secondary pathways could be theoretically estimated—but perhaps not experimentally verified—for various classes of allelochemicals. In that case, the partial metabolic cost could be combined with the theoretical cost of maintaining pathways and storage structures to yield a complete estimate of the metabolic cost. As discussed previously, the metabolic approach provides detailed information regarding secondary metabolism. However, it does not necessarily provide a complete measure of the related ecological consequences and, ultimately, plant fitness (see Chew and Rodman, 1979; Bazzaz et al., 1987; Fagerstrom, 1989; Zangerl and Bazzaz, 1992). As a result, the metabolic approach complements, rather than replaces, the more commonly used ecological approaches. Information regarding the dynamics of secondary metabolic processes, such as that provided by the technique we describe here, allows investigators to better evaluate the assumptions and predictions of current ecological theories by moving beyond "standing crop" measurements of allelochemical concentration (also see Gershenzon et al., 1993; Baldwin et al., 1994, Baldwin and Ohnmeiss, 1994).

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Journal of Chemical Ecology, Vol. 24, No. 3, 1998

Book Review

Biological Control of Weeds and Plant Diseases: Advances in Applied Allelopathy. Elroy L. Rice. Norman, Oklahoma: University of Oklahoma Press, 1995. US $55.00 hardback, 439 pp. ISBN 0-8061-2698-1

As a consequence of increased public concern for environmental damage caused by continued use of conventional synthetic agrichemicals, sustainable agriculture has gained great importance. Control or integrated management of weeds and plant diseases is viewed as a component of sustainable agriculture. During the past decade considerable research activities in agriculture have been directed toward biological control of plant diseases and weeds. To further stimulate research in this area, there was a need to bring these research results together and this book fulfills that need. It is an excellent source of reference for scientists involved in applying allelopathy to sustainable agriculture. More than 80% of 785 references are from those published after 1984, the year the author's other book, *Allelopathy*, 2nd edition (well received by the professionals), was published. The book emphasizes applied aspects of allelopathy. An important feature of the book is the addition of a "conclusion" section at the end of each chapter.

Chapter 1 is both the introduction and has a discussion of the allelopathic effects of crop plants. The introductory material could have been placed in a separate chapter to maintain uniformity in chapter organization.

The first two chapters abound with examples of crop plants affecting themselves, other crop plants and weeds, and weeds affecting crop plants and other weeds. The author strongly recommends a return to the practice of crop rotation to minimize or eliminate autotoxicity of crop plants; crop rotation design should be based on scientific information. Chapter 2 also includes examples of isolation, identification, and quantification of allelochemicals (including volatiles) from weeds, fresh or partially decomposed. He invites agricultural scientists to develop a sound strategy for weed management through intensive allelopathy research.

Chapter 3 discusses the role of microorganisms in plant growth. Included in this chapter are antibiotic production by microbes and their uptake by plants, phytotoxins produced by microbes and their impact on seed germination and plant growth, and growth promotion of plants by microbes. Several examples of the influence of rhizobacteria with special reference to *Pseudomonas* sp. are

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mentioned. Microbial biosynthesis of plant auxin is left out. Interaction between microorganisms other than in relation to biological control of plant diseases is evidently a neglected area of research. The author discusses this topic briefly only to stimulate research in this area.

Chapter 4 consists of four subsections. The first discusses general methods of biological control of weeds—crop rotation, breeding of crop plants resistant to weeds and allelopathy (mulch, plant pathogenic microbes, and phytotoxins of microbial and plant origin). The second subsection provides examples of weed control by cultural practices (cover crop, crop rotation, and mulch of carefully selected allelopathic plants). Extraction and identification of allelochemicals from allelopathic plants are mentioned in most examples. Weed control by plant pathogens and phytotoxins from microbial metabolites and allelopathic plants are discussed in the third and fourth subsections, respectively.

Chapters 5, 6, and 7 deal with biological control of plant diseases. Traditionally, this is the domain of plant pathologists, and the researchers involved in allelopathy generally avoid or neglect this area of research. Of late, however, plant pathogens as agents of weed control have attracted attention. The allelopathic effects of woody forest trees affecting understory herbaceous plants and regeneration of forest tree seedlings occupy the greater portion of Chapter 8.

Rice has done pioneering work by inviting plant pathologists to include allelopathy as a tool to control plant diseases and allelopathy researchers to consider biological control of plant diseases as another area of their research. In other words, Prof. Rice is asking the agricultural scientists to develop a holistic approach to address all agricultural problems instead of discipline-oriented research. Plant pathologists will find up-to-date references on biological control of plant diseases. In most references the author has mentioned experimental methodology in detail, which could have been abridged.

Overall, the book is an excellent source of reference for agricultural scientists interested in a multidisciplinary approach in solving agricultural problems.

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Book Review

Insect Pheromone Research: New Directions. Ring T. Cardé and Albert K. Minks (eds.). New York: Chapman & Hall, 1997. US $94.95, ISBN 0-412-99611-1

The first published insect pheromone identification was in 1959, and identifications have now been made from more than 1500 species. Many of these pheromones are used in monitoring pest insects, and populations of a few species have been controlled with pheromones or analogs by disruption of mating. Still other practical uses of pheromones have been demonstrated.

Although identifications continue, much of the current thrust in the pheromone field is to understand the neurophysiology of sensory perception and the integration of that information into command output. Insect work on neural processing is at the cutting edge of sensory physiology and provides models for work in many other groups, including vertebrates. In addition, research on hormonal regulation of pheromone biosynthesis and release is continuing, as is development of better monitoring tools to determine the concentration of pheromone under field conditions as a means to improve the practical use of pheromones or analogs in population control.

In order to bring the diverse researchers who work in this rapidly changing field together, the First International Symposium on Insect Pheromones was held in 1994 in Wageningen, The Netherlands. *Insect Pheromone Research: New Directions* contains the proceedings of that meeting. Reviews as well as original research are included in the five divisions of the book.

Part I, Control of Pheromone Production, contains one chapter on pheromone production and biology in cockroaches, two chapters on moths, and eight short chapters on mode of action of PBAN in several different insects. The section closes with a concise summary of the high points from Part I.

Part II, Sensory Processing of Pheromone Signals, contains chapters on molecular mechanisms of pheromone reception, pheromone-binding proteins and signal transduction, coding mechanisms for pheromone signals with comparative examples from moths, and two detailed chapters on the structure and function of the antennal lobe of the deutocerebrum into which olfactory neurons project. This section concludes with a chapter on neuroendocrine factors regulating male moth responsiveness to sex pheromone, followed by a summarizing chapter.

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Part III contains papers describing the neuroethology of pheromone-mediated responses. The opening chapter contains a discussion and review of the characteristics of odor plumes, their discontinuous concentration or pulsed structure, and measurement methods. A chapter on computer modeling of insect response to odor plumes follows and is a lead in to six chapters describing various and often controversial aspects of odor-modulated optomotor anemotaxis. The various authors do not always agree on whether male moths are most responsive to the pheromone blend or to certain, usually major, components in the blend. The structure of pheromone plumes, discontinuities and frequency of pulses, concentration, and blend are discussed as they influence optomotor anemotaxis, casting or zig-zag flight, and straight upwind flight. Not only moths, but birds and fish, follow odor trails by zig-zag pattern movements.

Part IV, Use of Pheromones in Direct Control, contains 11 chapters and begins with a good summary of mechanisms of mating disruption. There are three chapters on the use of EAG and single-sensillum recordings to measure pheromone concentrations under field conditions during disruption experiments. These chapters provide a good review and discussion of these electrophysiological techniques and what can be learned with them about pheromone distribution in a field. Chapters follow on disruption work with codling moth, tortricids in European vineyards and in apple orchards in New Zealand, pink bollworm, tomato pinworms and other insects in vegetable crops, bark beetles, and aphids.

Part V, Evolution of Pheromone Communication, contains nine chapters discussing the advances and problems in the evolution of pheromone communication in New Zealand tortricids, Trichoptera and primitive Lepidoptera, scarab beetles, and the cabbage looper. There are three chapters on the genetics of pheromone communication and a chapter on use of modeling population genetic simulations to assist in interpreting pheromone evolution. This section, like each of the others, concludes with a summarizing chapter.

The list of References cited in the text is extensive, with about 1500 complete citations given. A high proportion of these citations are from the 1990s, with many very recent citations. It is an excellent compilation of a very voluminous literature. There is an index that includes entries of topic and scientific name.

This book will be indispensable to those working in the pheromone field and to workers in neuroethology and behavior. It is a valuable source of information for those who teach insect ecology, chemical ecology, and insect physiology.

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Journal of Chemical Ecology, Vol. 24, No. 3, 1998

Erratum

In the paper "Monoterpene Composition of *Pinus sylvestris* Varieties Resistant and Susceptible to *Dioryctria zimmermani*" (Volume 23, no. 8) by C. S. Sadof and G. G. Grant, the monoterpene referred to as α-terpinolene should be replaced with the correct name α-terpinene six lines from the bottom of the abstract on p. 1917, six lines from the bottom of p. 1921, and in both places where it occurs in Table 1.

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FIELD TESTING SYNTHETIC PREDATOR ODORS FOR ROOF RATS (*Rattus rattus*) IN HAWAIIAN MACADAMIA NUT ORCHARDS

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Abstract—Field trials were conducted to determine whether the synthetic predator odors 3,3-dimethyl-1,2-dithiolane (DMDIT) and (*E*,*Z*)-2,4,5-trimethyl-Δ^3-thiazoline (TMT) were effective at eliciting a behavioral response in wild roof rats (*Rattus rattus*). The study site was a Hawaiian macadamia nut (*Macadamia integrifolia*) orchard with a recent history of roof rat feeding damage. The synthetic predator odors were encapsulated in urethane devices secured to tree branches. Mark–recapture data from live-trapping of rats and radio telemetry location data were used to assess behavioral responses of rats to the predator odors. Mark–recapture data indicated that DMDIT and TMT had no effect on capture numbers, reproduction, or body weight of rats. There was some indication that distribution of captures and number of locations relative to treated trees in TMT areas were less than in controls, but this pattern was not significant. The predator odors had no effect on home range or median distance from center of activity (MDIS) of rats as measured by telemetry. There was a trend of increasing values of MDIS on TMT areas in session 1 but not session 2. Overall we could not detect significant differences or consistent trends in responses of rats to DMDIT or TMT in these field trials.

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Key Words—Field bioassay, macadamia nut orchards, *Macadamia integrifolia*, Hawaii, roof rats, *Rattus rattus*, 3,3-dimethyl-1,2-dithiolane, (*E*,*Z*)-2,4,5-trimethyl-Δ^3-thiazoline, radio telemetry, mark–recapture, populations.

INTRODUCTION

The roof rat (*Rattus rattus*) occurs in a wide variety of habitats throughout the state of Hawaii. Its reputation as an adaptable generalist is apparent on this Pacific island by its presence in wooded gulches and forests, agricultural crops, and human structures (Tomich, 1986). *Rattus* species have a history of rapid colonization following their initial introduction to many Pacific islands (Atkinson, 1985; Buckle and Fenn, 1992). *Rattus* damage to the native flora and fauna as well as to food crops and food storage areas has been well documented (Stone, 1985; Tomich, 1986; Buckle and Fenn, 1992; Lund, 1994). Besides posing a risk to native fauna through direct consumption (i.e., plants, bird eggs, insects), rats are also capable of out-competing other animals with similar food sources (Clark, 1980). The roof rat has contributed to the rapid decline in native bird species on the islands, as this is the only rodent species that regularly utilizes tree canopies (Atkinson, 1985; C. P. Stone, personal communication).

The detrimental effects of rat populations on Hawaii's threatened flora and fauna are causing concern. Various agricultural growers are also concerned about feeding damage by rats. Sugarcane, macadamia nut, and coffee orchards have been experiencing rodent damage problems for several years in Hawaii (Tomich, 1986; Tobin et al., 1990, Tobin, 1992; Sugihara et al., 1995). Research into methods to control rats has been extensively investigated by the National Wildlife Research Center (NWRC) Hawaii Field Station and the Hawaiian Sugar Planters' Association.

Past attempts to control roof rat numbers in site-specific areas with toxicants have met with limited success. The capacity of *Rattus* spp. to withstand rodenticide poisoning attempts can be attributed to their neophobic nature, physiological resistance, social structure, and high reproductive rate allowing rapid reinvasion (Prakash, 1988). Other factors contributing to their resiliency in the Hawaiian Islands are their ability to breed year round combined with abundant food sources.

In previous field studies, predator odors were effective in the management of various small mammal species (Sullivan et al., 1988a–c). Field trials with predator odors were usually preceded by smaller-scale studies, such as an arena or pen trial, to initially determine if the animal of concern would respond. Once a desired response was observed, a field trial would then be suggested or performed. To date, most studies investigating predator odors have focused on mammals native to North America. The promising results in many of these

studies suggest a similar approach may provide a management technique for the roof rat in Hawaii.

Laboratory studies have indicated that rats display a fear response when exposed to synthetic predator odors (Vernet-Maury et al., 1984, 1992). The laboratory trials performed prior to this study (Burwash et al., 1998) indicated that the most promising odor for eliciting an avoidance behavior in the Hawaiian roof rat was 3,3-dimethyl-1,2-dithiolane (DMDIT). (*E*,*Z*)-2,4,5-Trimethyl-Δ^3-thiazoline (TMT) and 4-mercapto-4-methylpentan-2-one (MMP) also seemed to produce an avoidance response in the laboratory trials. However, of these two latter compounds, TMT was selected for field testing as it was also the odor that generated the greatest fear response in Wistar lab rats (Vernet-Maury et al., 1984) and wild-caught Norway rats (*R. norvegicus*) (Vernet-Maury et al., 1992).

Tobin et al. (1996) used radio telemetry to examine roof rat movement patterns within a macadamia orchard. Most of the rats den underground in the porous lava substrate or build nests from leaf clippings in the tree canopy. Rats in the orchard have a definite nocturnal feeding schedule with the greatest number of animals leaving their den sites by 23:00 hr. Lunar cycles and rainfall did not seem to affect this feeding pattern. Tobin et al. (1995) found that roof rats avoid traps scented with mongoose urine and feces in field trials, suggesting that the potential for odor avoidance exists.

Based on the laboratory results of Burwash et al. (1998) and other small mammal field study results, we predict that roof rats will avoid predator odors in the field. This study was designed to test the hypotheses that predator odors would: (1) reduce the number, incidence of breeding, and body weights, of roof rats captured; (2) increase the mean maximum distances moved (MMDM) between subsequent captures (mark–recapture) and median distances from center of activity (MDIS) (telemetry) for roof rats; (3) increase the home range size of roof rats; and (4) reduce the proportion of roof rat locations in treated trees.

METHODS AND MATERIALS

The effectiveness of predator odors at producing a response in the roof rat was determined with mark–recapture and radio telemetry techniques. Many studies have also gathered useful information on changes in small mammal populations with mark–recapture techniques (Krebs, 1966; Ritchie and Sullivan, 1989; Sullivan, 1990). Research specific to the roof rat has used live-trapping techniques as well as radio telemetry analysis (Chin, 1983). The design and sampling methodology used for our study were based on previous mark–recapture studies, and a pilot field trial was also conducted to test the radio transmitter collars and recapture success with various baits. The field study was conducted in two sessions based on the battery life of the radio transmitters: session 1 from June

to August 1994 and session 2 from September to December 1994. The radio telemetry procedure remained the same for both sessions; however, the mark–recapture methodology was modified slightly between sessions as discussed below.

Study Site. The study site was a 999-ha macadamia nut orchard located 15 km south of Hilo in the state of Hawaii. The orchard was on the windward side of the island of Hawaii where rainfall was substantial (>1500 mm/yr) and the general topography relatively flat. The majority of the orchard was comprised of ~25-year-old macadamia trees of different varieties. This varietal mix is primarily for pollination purposes. Orchard soils were volcanic with a 0.3-m layer of crushed lava on the surface providing the substrate in which the macadamia trees were planted. The porous nature of the lava beneath this crushed layer provided rats with an extensive tunnel network easily accessible through many openings to the surface. Vegetative ground cover throughout the orchard was minimal as a result of the regular use of herbicides and manual clearing of leaves. The orchard was laid out in blocks (mostly rectangular) separated by gravel access roads on all sides and Norfolk Island pine tree (*Araucaria heterophylla*) windbreaks on at least two sides of each block. These windbreak areas had a very deep duff layer composed of fallen debris and orchard trimmings that provided another denning area for rodents.

This orchard was chosen to test the predator odors based on the recent history of rodent damage recorded in the macadamia orchard (Tobin et al., 1993) and the relative homogeneity of the orchard. This homogeneity provided a large area of very similar blocks in which to replicate treatments. The blocks were composed of the same tree variety ratio (variety 660, 86%: 508, 9%: 212, 5%) and trees were also of similar age (20–25 years), height (8–10 m), and density (240 trees/ha). Three 20-ha blocks were relatively flat and each block was separated by at least 200 m. Each of the three blocks was divided into two 4-ha rectangular (160 × 250 m) grids. This allowed the study to focus on animals living primarily within the blocks and to avoid those individuals utilizing the windbreaks. Each grid was at least 20 m from the road edge and at least 300 m from the adjacent grid in the same block. As the trees were planted in a grid layout (6.5-m × 6.5-m spacing), specific row and tree locations could be assigned to every tree.

This six-month study was separated into two periods: session 1 from June 13 to August 31, 1994, and session 2 from September 19 to December 14, 1994. For both sessions, pretreatment information was gathered for both the mark–recapture and telemetry information.

Mark–Recapture. In session 1, 100 live-traps (80 Hagaruma, 20 Tomahawk) were used on each grid with placement on every three to four trees per row on every other row pair within each 4-ha grid. Traps were placed on large

lateral branches 1–2 m above ground because previous live-trapping had greater capture success at this location. Placing the traps on the ground tended to capture more mongoose, and those that did capture rats on the ground had increased trap deaths as a result of mongoose predation. All traps were cleaned prior to use and were secured to the branch with nylon twine and rubber bands.

Prebaiting was carried out three days prior to the first trap day of a given trapping week to allow animals to become familiar with taking bait from the traps. This was accomplished by locking open the traps and placing a coconut chunk smeared with peanut butter inside. On the initial trap day, the traps were rebaited and set during the day, left open throughout the night, and checked the following morning. Each trapping week was comprised of three nights of trapping following prebaiting, with trapping taking place every three weeks.

All captured animals were identified to species and marked with an individually numbered ear tag. Color of pelage, sex, weight, and breeding condition (males: scrotal/abdominal; females: perforate/nonperforate and pregnant/not pregnant) were recorded for each animal. An open-ended mesh net-bag with a rope cinch was used to handle each animal for data collection and ear-tagging.

The only mark–recapture data analyzed were those from session 1 as the design in session 2 yielded too few captures to provide a worthwhile comparison. As the numbers of trapped animals were quite variable on each grid and the duration of trapping was limited to five weeks in session 1, open population estimates were applied with caution. Information on composition of rat populations was gained, however, and this provided some useful insight about changes within the captured populations.

In session 2, the above mark–recapture design was used to collar the animals, after which the trap layout was modified. Because of the low number of traps in treated areas in the first session, the design was modified to focus trap placement in treated trees for session 2. Once the areas to receive treatment were determined (see odor placement section), 10 traps were placed within the treated area and trapped on the same schedule and procedure as in session 1.

Radio Telemetry. Roof rats were initially captured in live-traps in each of the six study grids with the session 1 mark–recapture design. Only adult rats were used for telemetry to maintain a similar age class and sufficient sample size. To ensure that radio-collar weight would have minimum effect on normal behaviors, no animals under 90 g were used for radio telemetry. Six animals (three males and three females) were initially radio-collared on each grid. The animals were anesthetized with a general anesthetic (Metofane) by placing the individual into a sealed plastic container lined with anesthetic-treated ($\sim$ 10 ml) cotton. Within 5–10 min the animal would be sedated enough to handle safely. The individual would then be processed as in the mark–recapture procedure, and fitted with a radio transmitter neck collar (Holohil PD-2C). Before releasing

the animal, the transmitter signal would be checked and the animal placed back into the trap to recover. Usually 10–15 min following the collaring, the animal would fully recover and be released at the point of capture.

As roof rats are primarily nocturnal, most of the telemetry locations were taken during the night. A telemetry week consisted of four days of locating animals with each telemetry day comprised of one day location (12:00–17:00 hr) and three night locations (19:00–21:00; 21:01–23:00, and 23:01–01:00 hr). This design was based on the number of active radio-collars and the number of observers available. An observer was equipped with a headlamp, a portable radio receiver (Custom Electronics of Urbana Inc. or Wildlife Materials Inc.) and a hand-held yagi antenna (Wildlife Materials Inc.) to locate radio-collared rats. During an individual's location, the animal would be tracked to a single tree with its location either above or below ground determined. The specific information recorded was: observer, date, time, receiver, tuning and signal strength, location (tree/underground/surface), activity (moving/stationary), visual confirmation (yes/no), and general weather conditions (wind, rain, cloud cover, lunar phase). A shortened data label would then be transcribed onto flagging tape and secured to the appropriate tree. At the end of the four-day telemetry week, the exact location (row and tree label) for each flag was determined and recorded before flag removal.

During each telemetry night, the order in which grids were sampled and specific rats tracked was systematically shifted during each 2-hr location period. This would ensure that individual rats were not always being located at the same point within each 2-hr period.

Predator Odor Semiochemicals. The chemical compounds to be tested as repellents were originally derived from predator species, commonly from the anal scent gland, urine, or feces. The compounds have generally been identified either from extracts of these secretions or from the volatiles that emanate from them. The components believed to have semiochemical activity were prepared synthetically, albeit as racemates. The synthetic odor was encapsulated in a release device (usually PVC or urethane) to control release rates and protect the chemicals from excessive exposure during field use (Sullivan et al., 1990). The synthetic odors were synthesized by Industrial Research Limited, New Zealand, and Phero Tech Inc., Delta, British Columbia, Canada, then encapsulated in release devices by Phero Tech. A list of the odors, an abbreviation, and their original source are given in Burwash et al. (1998). The DMDIT (3,3-dimethyl-1,2-dithiolane) devices were loaded with 8 mg of active ingredient in a 3-cm urethane device, while TMT [(*E*,*Z*)-2,4,5-trimethyl-Δ^3-thiazoline] devices were loaded with 10 mg of active ingredient in a 6-cm urethane device. The difference in concentration of active ingredient was a result of the amount of synthetic chemical available at the time of the study. As the release devices had an expected field life of three weeks, they were replaced once, after the third week

following treatment application, within each session (six treatment weeks per session).

Experimental Design. Each treatment (DMDIT, TMT, and control) was replicated twice and randomly assigned to the six grids. The application of the treatment was focused in areas specific to individual animals rather than in a broadcast area design. This was decided primarily because of the reliable individual movement data available with the telemetry procedure. We were also limited by the number of odor repellents available and personnel to apply the treatment. Focusing on the individual animals allowed assessment of whether individuals shifted their activity in response to placement of the semiochemicals.

After two weeks of pretreatment telemetry locations, specific areas to be treated were determined. Every animal on a grid would receive the same treatment odor even though the treated areas were not continuous over the entire grid but concentrated in specific areas for each animal. This was to prevent any possible contamination of different treatments within a grid. The design in session 2 was modified following the results from session 1.

In session 1, treated areas were composed of nine adjacent trees within an individual rat's weekly home range area, based on frequency of locations during the pretreatment period. The general shape of the treated area was a three-tree by three-tree square. However, due to missing or dead trees, this shape often varied. To maintain consistency in the treated areas, the treatment trees had to be adjacent to at least one other treated tree.

The predator odors were applied by placing a coated wire through a hole in each repellent device and twisting the wire ends to form a loop. Flagging was then used to secure the device to tree branches. Each treated tree received eight odor devices placed at varying heights (2–4 m) throughout the canopy. Their location was also dispersed relative to the main trunk of the tree. Generally, four devices were placed distal to the trunk and four were placed proximal, at variable heights above ground. Feeding by rats was often localized, as indicated by gnawed shells and husks found in flat pocket areas at the base of large branches. These locations and obvious runway areas (along larger branches) were treated with repellent devices. In session 1 there were 72 devices per area (per radio-collared rat).

The schedule was designed such that telemetry weeks occurred on the first and third week following initial treatment application and, following reapplication, mark–recapture trapping took place in the weeks between telemetry sessions.

After mapping the results from session 1, it was apparent that the treatment area was quite small relative to the individual's weekly home range, which often led to the rats avoiding the "treated" (marked but not treated) trees on the control grids. During session 2 we expanded the treated area to 16 trees and increased the number of odor devices placed in each tree to 12 [192 devices per

area (per radio-collared rat)]. A further modification of the application was to place four of the 12 devices around the trunk at a height of ~0.5 m.

This session was also divided into two treatment periods and the first period (session 2, treatment 1) used the same treatment designation as in session 1. Following two weeks of posttreatment telemetry, the semiochemicals were removed. After a week delay, the second period (session 2, treatment 2) was initiated with a pretreatment telemetry week followed by two weeks of posttreatment monitoring. For the second treatment, semiochemical applications were systematically shifted so that each rat received a different treatment for session 2, treatment 2. The design was balanced so that each treatment 1 group would be divided so that each half would receive different semiochemicals for treatment 2. The intent was to observe any trends in individual response to a new semiochemical treatment.

Statistical Analysis. For the mark–recapture data, comparisons in the number and composition of the captured individuals were generated. The number of captures were totaled by week and separated into proportion of recaptures for comparison. Comparisons were made with the actual capture information per unit effort, and other population parameters, by using randomization testing (RANDMIZE program) (Manly, 1990). Randomization techniques are designed for detecting nonrandom change in studies with little or no replication of experimental units and for paired time-series data from individual treatment and control systems (Carpenter et al., 1989). This methodology is not bound by the assumptions of parametric statistics (random sampling, normally distributed populations, and equal variances) as the technique determines the error distribution of its test statistics by randomly reordering the data set (Carpenter et al., 1989; Manly, 1990).

Mark–recapture data were also used to compare capture success within specific treated and untreated areas (capture success within/adjacent to treated trees). This information could also be compared to results from radio telemetry techniques. These data were grouped among treatments because of the low number of traps within or adjacent to treated areas.

The number of recaptures by individual was insufficient to provide a meaningful home range estimate from the mark–recapture data. Where sufficient replication existed, analyses of variance (ANOVA) were conducted to compare between treatments by trapping week.

We calculated the minimum area convex polygon (MCP) for each radio-collared rat to estimate its weekly home range size and median distance from center of activity (MDIS). MDIS was calculated as the median distance of all locations for an animal from its center of activity (mean x and y coordinates of all locations) (SAS Institute Inc.), MCP was calculated with McPaal Microcomputer Programs for the Analysis of Animal Locations (Stuwe and

Blohowiak, 1989). In order to calculate home range estimates, a minimum of 14 locations was needed based on a prestudy plot of home range size versus number of locations.

We also calculated the proportion of telemetry locations in treated trees from the total number of in-tree locations. The capture success relative to treatment areas was determined by considering the proportion of captures in traps located within or adjacent to (defined as within 1 adjacent tree) treated areas. This information should indicate whether an individual is selecting specific trees within its weekly home range.

Separate two-way repeated-measures ANOVAs for the telemetry variables were performed to compare between pre- and posttreatment and between treatments. Sex was grouped, as previous telemetry work in this orchard found no difference in home range estimates between sexes (Tobin et al., 1996b). All ANOVAs were conducted with alpha (α) set at 0.05. To make multiple comparisons, Duncan's multiple-range test was used with an experiment-wise error rate of 0.05 (Saville, 1990).

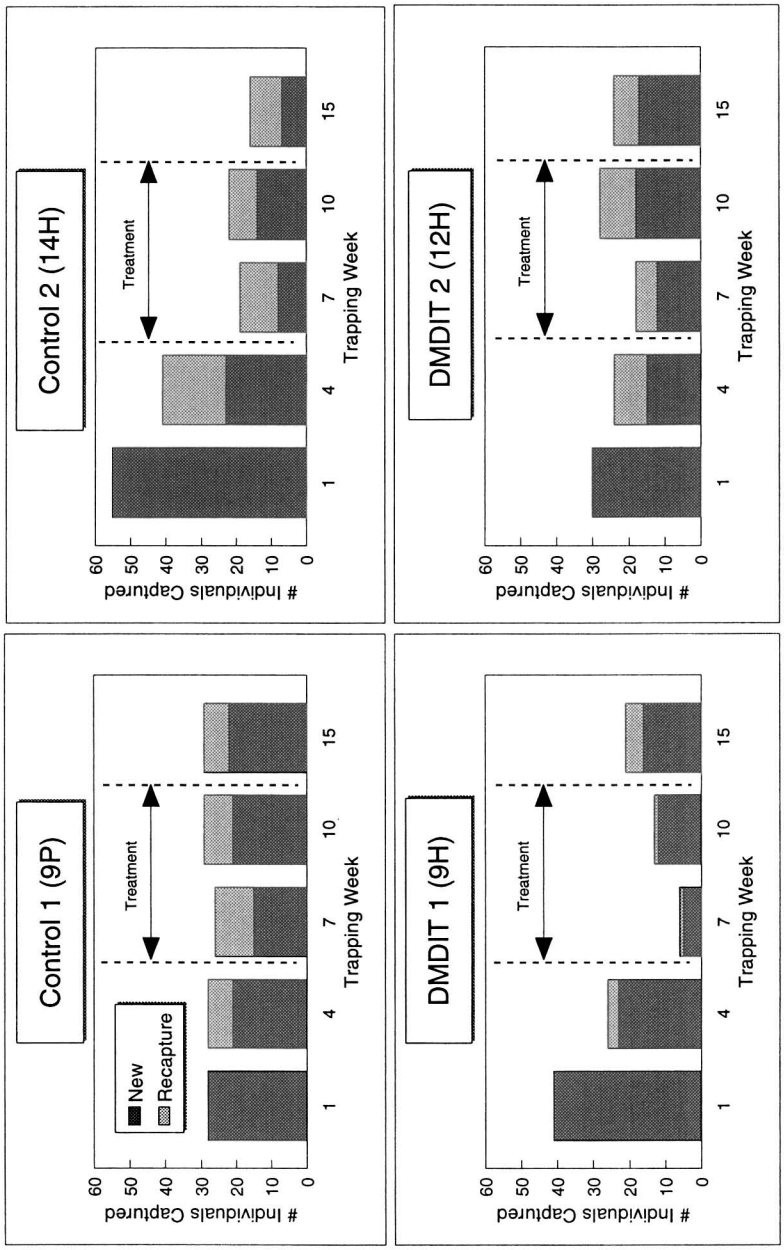
RESULTS

Mark–Recapture. Total number of captures for a trapping week ranged from 5 to 55 individuals. The number of captures and recaptures by grid is displayed in Figure 1. There was a balanced sex ratio for all populations throughout the study. Overall, 582 individual roof rats were captured a total of 1089 times during five weeks of live-trapping. By treatment, 214 roof rats were captured on the control grids, 189 captured on the DMDIT grids, and 179 captured on the TMT grids.

The relative capture data were tested through randomization to compare capture numbers per 300 trap-nights between treatments and between pre- and posttreatment. There were no nonrandom differences in capture numbers of roof rats in pairwise comparisons of controls and DMDIT treatments (two-tailed, $P = 0.47$), controls and TMT treatments (two-tailed, $P = 0.47$), or DMDIT and TMT treatments (two-tailed, $P = 0.74$).

The number of males in breeding condition did not change following treatment application (Figure 2). Randomization testing revealed no nonrandom differences in number of scrotal males captured for pairwise comparisons of controls and DMDIT treatments (two-tailed, $P = 0.71$), controls and TMT treatments (two-tailed, $P = 0.20$), and DMDIT and TMT treatments (two-tailed, $P = 0.50$).

There was no significant difference in the average weight of male roof rats between control and treatment grids during the pretreatment weeks (ANOVA,


Control 1 (9P)
Control 2 (14H)
DMDIT 1 (9H)
DMDIT 2 (12H)
New
Recapture
Treatment
Individuals Captured
Trapping Week
0
10
20
30
40
50
60
1
4
7
10
15

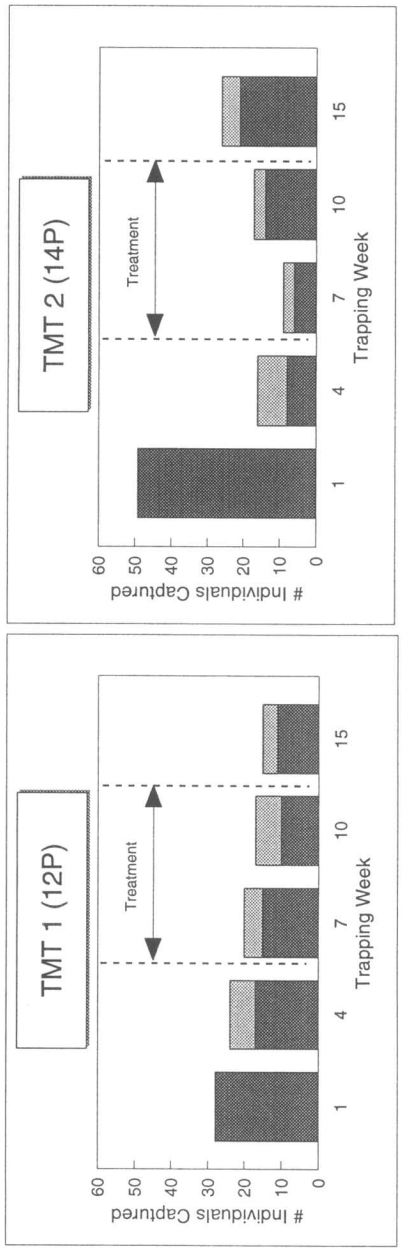
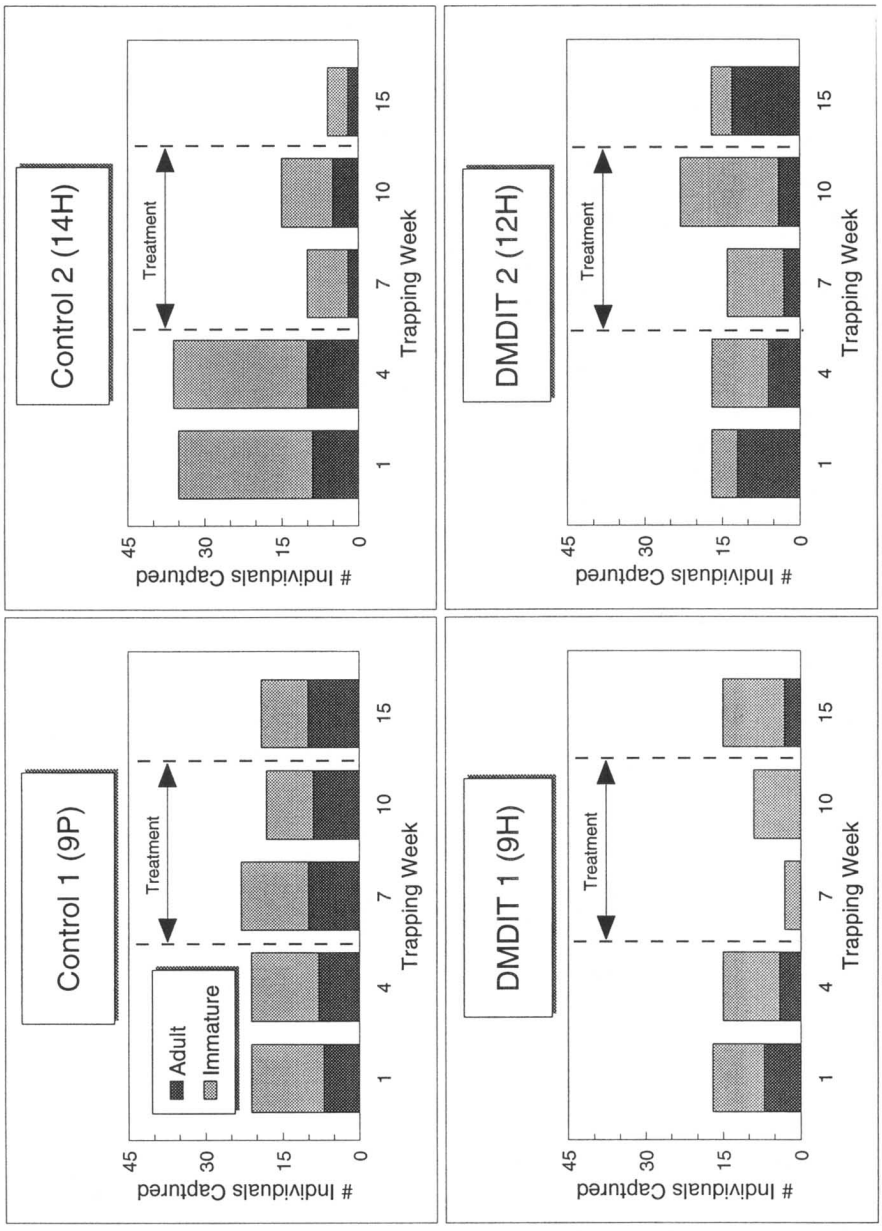


FIG. 1. Total number of roof rat captures and recaptures (numbers/300 trap nights) over five trapping weeks in session 1. Treatment period is indicated by vertical hatched lines and horizontal arrow.


Control 1 (9P)
Control 2 (14H)
DMDIT 1 (9H)
DMDIT 2 (12H)
Adult
Immature
Treatment
Individuals Captured
Trapping Week
0
15
30
45
1
4
7
10
15

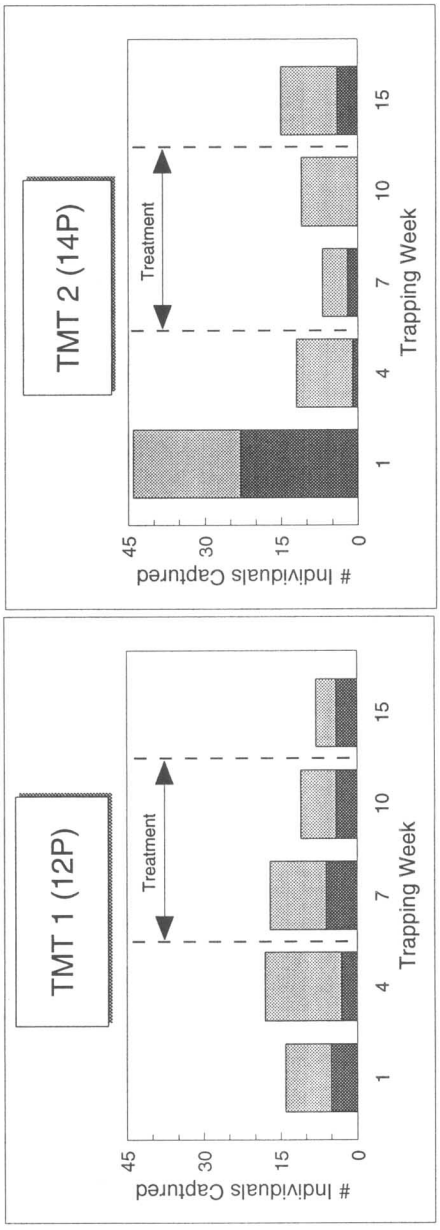


FIG. 2. Total number of individual male roof rats captured (numbers/300 trap nights) by age (all adults captured in breeding condition) in session 1. Treatment period indicated by vertical hatched bars and horizontal arrow.

week 1: $P = 0.69$, week 4: $P = 0.87$), during the treatment period (ANOVA, week 7: $P = 0.68$, week 10: $P = 0.48$) or during the posttreatment period (ANOVA, week 15: $P = 0.46$) (Table 1).

Mean maximum distances moved (MMDM) between consecutive trapping weeks are shown in Table 2. There were no significant differences in MMDM between treatments during pretreatment (ANOVA, $P = 0.77$) or following treatment application (ANOVA, week 4–7: $P = 0.69$, week 7–10: $P = 0.20$). Proportion of captures in traps within or adjacent to (within one adjacent tree) treated areas is displayed in Figure 3. Although capture numbers were quite low, the distribution of captures relative to the treated areas provided an indication of predator odor avoidance. Randomization testing detected no nonrandom differences in the proportion of captures within/adjacent to treated areas in pairwise comparisons of controls and DMDIT treatments (two-tailed, $P = 0.11$), controls and TMT treatments (two-tailed, $P = 0.85$), or DMDIT and TMT treatments (two-tailed, $P = 0.07$).

Telemetry. For all telemetry data, estimates were initially plotted for each animal for the duration of session 1. As rats displayed a high degree of individual variability in laboratory arena trials (Burwash et al., 1998), we felt it worthwhile to first display the results by individual. A consistent problem throughout session 1 was transmitter slippage or predation. Of the six rats initially collared on each grid, 0–3 rats per grid provided data throughout the entire session. Many of the radio-collared rats either had their transmitters recovered on the surface ($\sim 25\%$) (slip or predation), remained stationary underground ($\sim 15\%$) (slip or predation), or their signal was entirely absent ($\sim 5\%$) (transmitter failure or moved from grid >2 km). In one case a female rat lost her radio transmitter in the tree canopy two weeks after collaring. The radio transmitter was recovered ~ 5 m above the ground in working condition, with no signs of predation. One animal died from predation. This female weighed 120 g and was located during the first week following the initial DMDIT treatment application. The animal was first located in the canopy (19:00–21:00 hr), but in the subsequent location (21:01–23:00 hr), was observed on the surface running erratically. This activity appeared very unusual as rats were rarely observed on the ground in the orchard. In the final reading (23:01–01:00 hr) this individual was recovered on the surface missing half the lower body, with obvious signs of feral cat predation as evidenced by tooth puncture marks in the back of the neck and spine. To combine all individuals for each treatment would have yielded a widely varying sample size by week. It would also have been unreliable to use data from individuals not present throughout most of each session, as individual biases would not have been consistent across each telemetry week. Therefore, we decided to calculate average values only for those rats present throughout at least one pretreatment and one posttreatment (consecutive) telemetry week. In session 1 the number of rats (sample size) for all telemetry measurements was at least 3, except in

TABLE 1. MEAN MALE BODY WEIGHT OF ROOF RATS CAPTURED ON CONTROL, DMDIT, AND TMT TREATMENT GRIDS IN SESSION 1[a]

	Weight (g)									
	Pretreatment				Treatment				Posttreatment	
	Week 1		Week 4		Week 7		Week 10		Week 15	
Grids	Mean ± SE	*N*	Mean ± SE	*N*	Mean ± SE	*N*	Mean ± SE	*N*	Mean ± SE	*N*
Control										
9P	69 ± 10	21	70 ± 9	21	66 ± 6	23	77 ± 9	18	83 ± 11	19
14H	66 ± 8	35	67 ± 8	36	57 ± 10	10	67 ± 13	15	98 ± 35	4
DMDIT										
9H	76 ± 9	17	58 ± 9	15	34 ± 0	3	39 ± 2	9	59 ± 10	15
12H	102 ± 11	17	72 ± 12	17	57 ± 9	14	59 ± 7	23	108 ± 12	17
TMT										
12P	79 ± 16	14	55 ± 10	18	58 ± 9	17	81 ± 16	11	68 ± 18	8
14P	98 ± 8	44	47 ± 6	12	63 ± 14	7	39 ± 2	11	64 ± 12	15

[a]Values are mean weights with standard error (SE) and sample size (*N*); 9P, 14H, 9H, 12H, 12P, and 14P are grid names.

TABLE 2. MEAN MAXIMUM DISTANCE MOVED (MMDM) BETWEEN CONSECUTIVE TRAPPING WEEKS OF ROOF RATS CAPTURED ON CONTROL, DMDIT, AND TMT TREATMENT GRIDS IN SESSION 1[a]

	MMDM (m)					
	Pretreatment,		Treatment			
	week 1–4		Week 4–7		Week 7–10	
Grids	Mean ± SE	*N*	Mean ± SE	*N*	Mean ± SE	*N*
Control						
9P	10.8 ± 4.8	13	23.8 ± 6.5	13	17.4 ± 5.4	12
14H	30.4 ± 5.9	37	25.7 ± 7.1	25	28.9 ± 7.2	9
DMDIT						
9H	22.2 ± 4	19	20.9 ± 8	7	4.5 ± 4.5	3
12H	15.6 ± 3.6	18	23.7 ± 4.2	17	28.1 ± 5.6	13
TMT						
12P	17.6 ± 3.6	15	20.5 ± 6.1	13	10.3 ± 3.3	13
14P	27.5 ± 4.1	27	6.3 ± 3.1	11	8.3 ± 3.4	12

[a]MMDM is measured between first capture point in each of two successive trapping weeks. Standard error (SE) and sample size (*N*); 9P, 14H, 9H, 12H, 12P, and 14P are grid names.

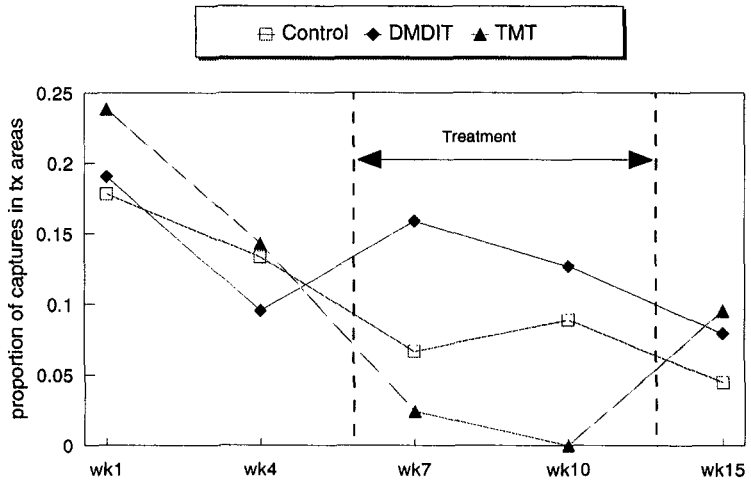


FIG. 3. Proportion of roof rat captures in traps within or adjacent (within 1 tree) to treated areas. Data are from session 1 mark–recapture and displayed by treatment type (defined in legend) by week. Treatment period indicated by vertical hatched lines and horizontal arrow.

pretreatment week 1 (not used in the analysis). For session 2, the number of rats (sample size) for all telemetry measurements was at least 7, except in the pretreatment week.

Session 1. The individual's weekly MCP home range estimates ranged from 63 m^2 to 4730 m^2 throughout session 1. The mean MCP home range estimates by telemetry week are displayed in Figure 4. A repeated measures ANOVA found no difference in treatments within or between weeks ($P = 0.61$). Median distance from the center of activity (MDIS) for individual roof rats ranged between 4 and 45 m. The mean MDIS estimates by telemetry week are displayed in Figure 5. Repeated measures ANOVA found no difference in treatments within or between weeks ($P = 0.45$). The proportion of telemetry readings in treated trees ranged between 0 and 85%. Mean estimates for proportion of

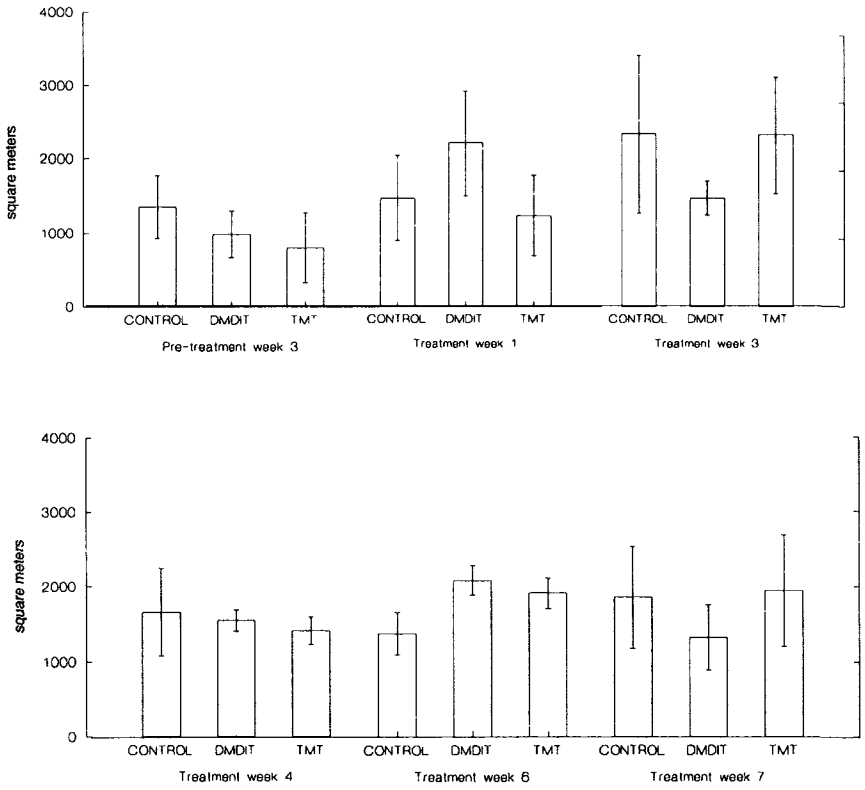


FIG. 4. Mean minimum convex polygon (MCP) estimates for three treatments (control, DMDIT, and TMT) by telemetry week during session 1 (June 15–August 31, 1994). Each value is the mean of at least three replicates ± standard error (SE).

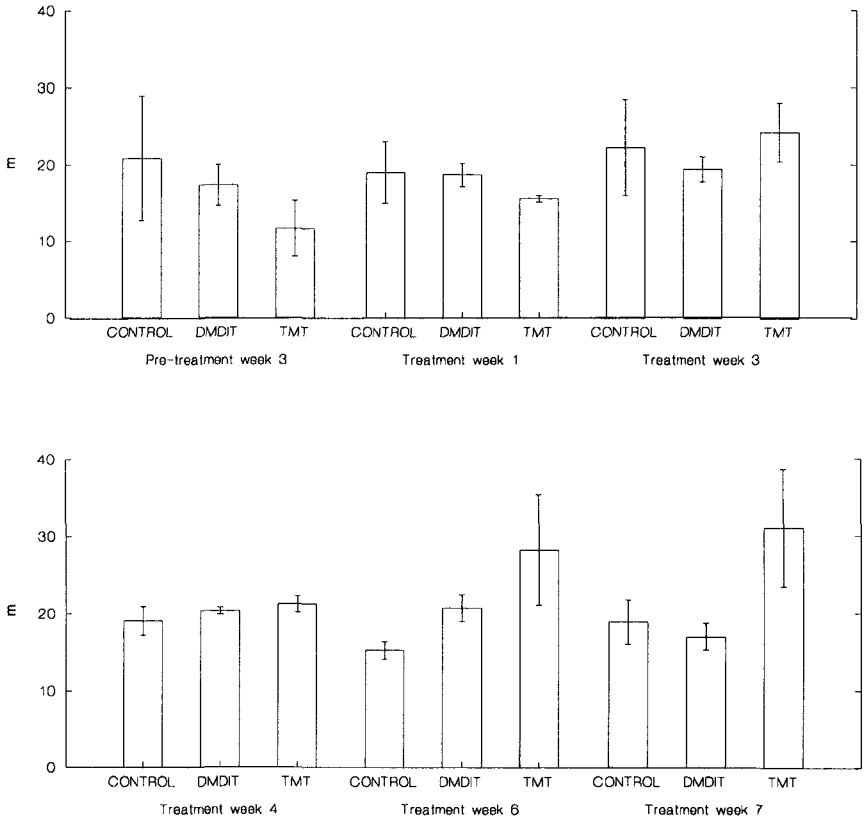


FIG. 5. Mean median distance traveled from center of activity (MDIS) estimates (meters) for three treatments (control, DMDIT, and TMT) by telemetry week during session 1 (June 15–August 31, 1994). Each value is the mean of at least three replicates ± standard error (SE).

telemetry locations in treated trees are displayed in Figure 6. Repeated-measures ANOVA revealed no difference in treatments within or between weeks ($P = 0.35$).

Session 2. The individual's weekly minimum convex polygon (MCP) home range estimates ranged from 125 m^2 to 12,162 m^2 throughout session 2. The mean MCP home range estimates for roof rats in session 2, treatment 1, are displayed in Figure 7. Repeated-measures ANOVA found no difference in treatments within or between weeks ($P = 0.54$). Mean MCP home range estimates for roof rats in session 2, treatment 2, are displayed in Figure 8. Repeated-

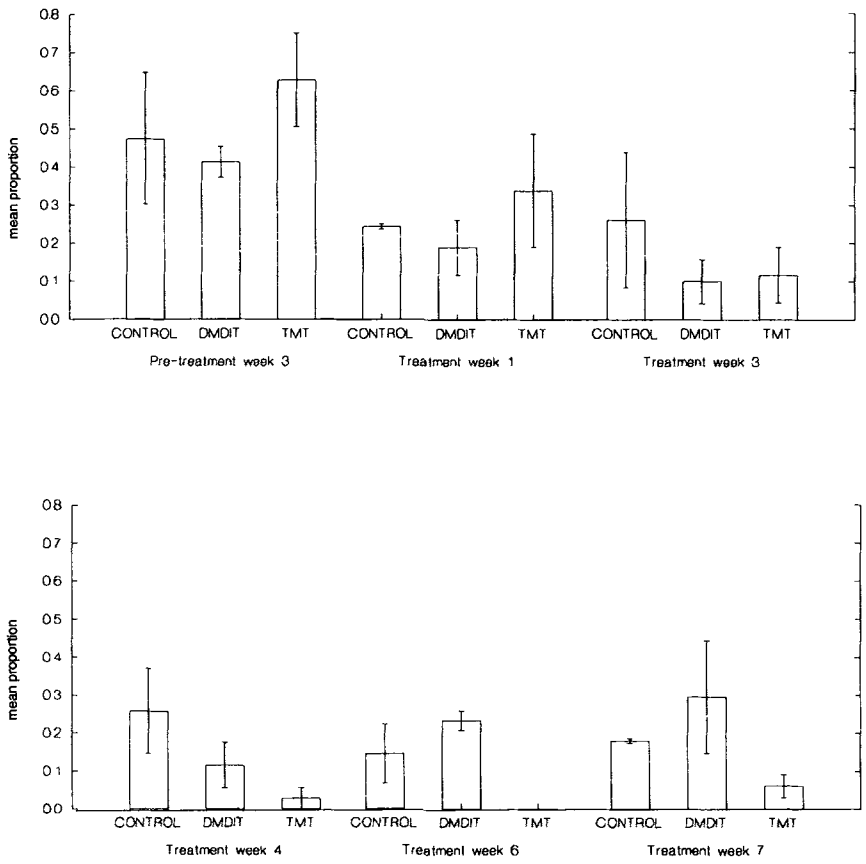


FIG. 6. Mean proportion of telemetry readings in treated trees for three treatments (control, DMDIT, and TMT) by telemetry week during session 1 (June 15–August 31, 1994). Each value is the mean of at least three replicates ± standard error (SE).

measures ANOVA found no difference in treatments within or between weeks ($P = 0.08$).

For individual rats, median distance from center of activity (MDIS) ranged from 5 to 60 m. No trends in groups of individuals were obvious from these data. Mean MDIS values for roof rats present throughout session 2, treatment 1, ranged from 16 to 26 m. No differences within or between treatment weeks were determined ($P = 0.34$). For session 2, treatment 2, mean MDIS values ranged from 14 to 23 m, and no significant differences were found ($P = 0.19$).

For individuals present throughout session 2, treatment 1, the mean pro-

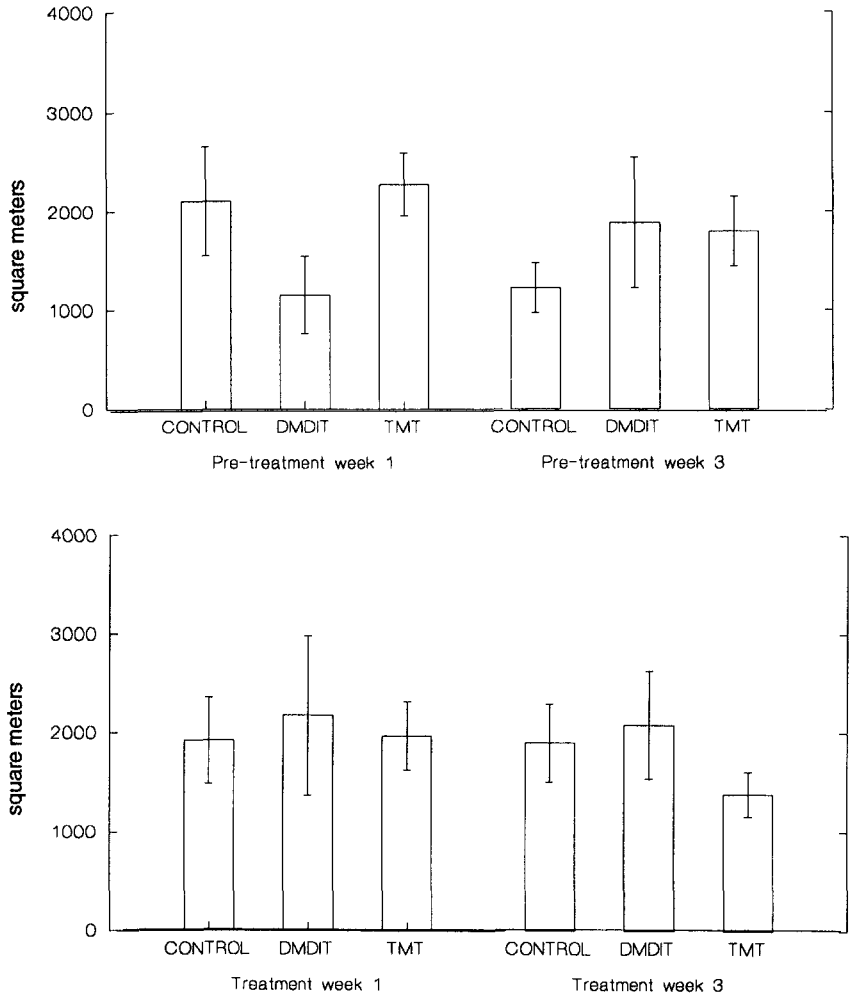


FIG. 7. Mean minimum convex polygon (MCP) estimates for three treatments (control, DMDIT, and TMT) by telemetry week during session 2, treatment 1 (September 20–November 4, 1994). Each value is the mean of at least seven replicates ± standard error (SE).

portion of readings in treated trees ranged from 25 to 57% (Figure 9). No significant differences were found within or between treatment weeks ($P = 0.53$). In session 2, treatment 2, the mean proportion of locations in treated trees ranged from 20 to 77% (Figure 10). Again, there were no significant differences found within or between treatment weeks ($P = 0.12$).

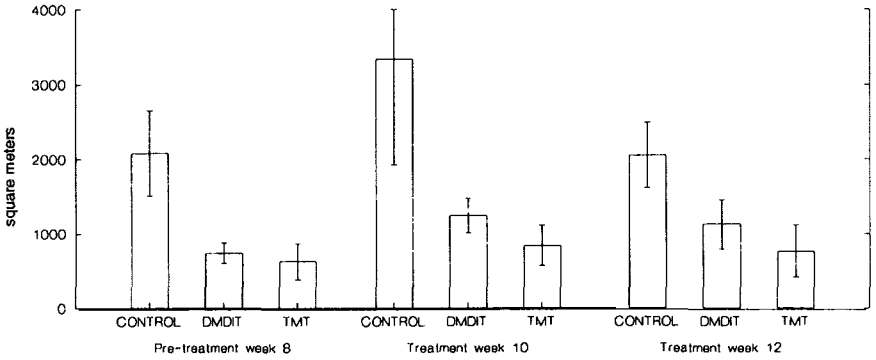


FIG. 8. Mean minimum convex polygon (MCP) estimates for three treatments (control, DMDIT, and TMT) by telemetry week during session 2, treatment 2 (November 9–December 10, 1994). Each value is the mean of at least seven replicates ± standard error (SE).

DISCUSSION

A concern throughout this study was the individual variability displayed by the roof rat. Ideally one could reduce this variability by increasing the sample size and number of replicates. However, the results from Burwash et al. (1998) suggested that two predator odor treatments should be field-tested, which limited the experimental design to two replicates for each of three treatments.

Variable capture rates indicated cautious interpretation of population parameters. However, some of the population parameters based on mark–recapture information provided worthwhile data as to changes in the trapped population. Many other small mammal population studies have recognized variable trappability when interpreting mark–recapture results (Sullivan, 1990, 1994; Nichols and Pollock, 1983).

Telemetry data were also subject to the effects of small sample sizes with a low number of replicates. Predation, radio-collar slippage, and malfunction all contributed to the small sample sizes of animals, especially towards the end of session 1. Although sample sizes were quite small, the specific individual results from the telemetry analysis provided useful insight into patterns of habitat use.

Poor capture success in live trap studies with rats is common (Worth, 1950; Kartman and Lonergan, 1955; Lindsey et al., 1973; Chin, 1983). Rats become trap-shy following initial capture (Lindsey et al., 1973; Spencer and Davis, 1950) while juvenile rats become trap-happy (Nichols and Pollock, 1983). With these concerns in mind, we used a mark–recapture design based on an earlier successful pilot study. An important technique in this procedure was to prebait

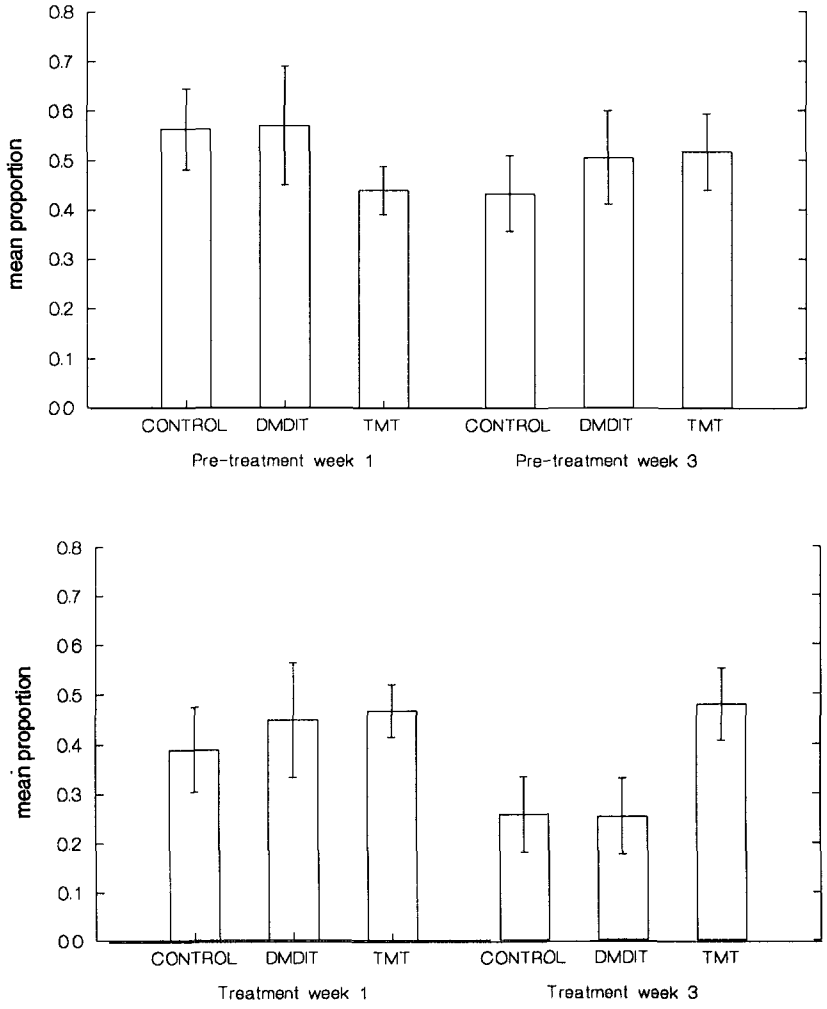


FIG. 9. Mean proportion of telemetry readings in treated trees for three treatments (control, DMDIT, and TMT) by telemetry week during session 2, treatment 1 (September 20–November 4, 1994). Each value is the mean of at least three replicates ± standard error (SE).

traps (locked open) starting four days prior to each trapping week. This should have reduced neophobic responses to the traps and to recapture. The methodology used for the pilot study followed small mammal mark-recapture studies in North America (Sullivan, 1990; Ransome and Sullivan, 1997). We also fol-

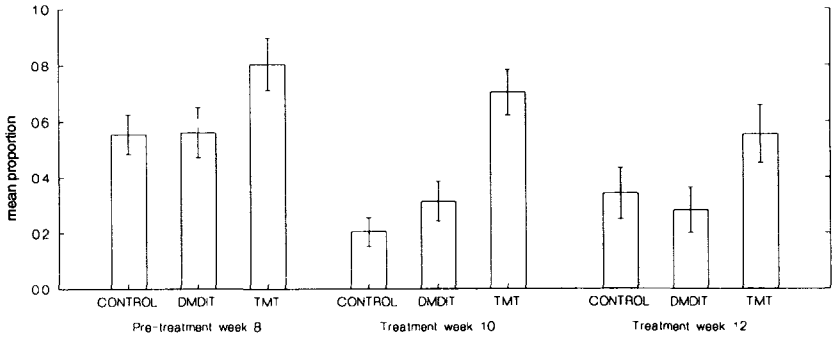


FIG. 10. Mean proportion of telemetry readings in treated trees for three treatments (control, DMDIT, and TMT) by telemetry week during session 2, treatment 2 (November 9–December 10, 1994). Each value is the mean of at least three replicates ± standard error (SE).

lowed standard operating procedures utilized by the National Wildlife Research Center (NWRC) to live-trap rodents in the orchard.

Comparisons between actual capture numbers (relative density) resulted in no significant effect of treatments on numbers of rats. Although capture numbers varied greatly, some useful information regarding the composition of these captures was gained. Because female breeding condition was more difficult to assess and weights fluctuated with pregnancy status, only males were considered for evaluation of breeding condition and body weight. The number of breeding males was not statistically different between treatment grids or between treatment weeks.

Mean male body weights did not vary within or between treatment weeks. Most of the declining trend in mean weights can be attributed to a greater proportion of juveniles being captured in weeks subsequent to the initial trapping week. Survival rate estimates were not tested because of low trappability estimates and the relatively short mark–recapture sampling period. Thus, we cannot accept hypothesis 1: reduction in number, incidence of breeding, and body weights of rats in predator-odor treated areas.

Previous mark–recapture studies with predator odors and small mammals have indicated movement of animals from treated areas (Sullivan and Crump, 1986; Sullivan et al., 1988a, b). In this study, the treated areas were in patches within the trapping grid, thereby resulting in an uneven distribution of treatment. The MMDM did not differ between treatments or between treatment weeks. MDIS telemetry data for session 1, although not statistically significant, indicated a trend of increasing values on the TMT grids. However, this trend was not observed with the session 2 telemetry data. Hypothesis 2, that predator odor

treatments would increase the distance traveled from an individual's center of activity, was not supported by our results.

Individual home range estimates varied greatly for both telemetry sessions. Hypothesis 3, that roof rat home range estimates will increase following predator odor treatment application, was not supported by our results. Although not assessed, MDIS and MCP estimates are probably highly correlated, as determined in a previous study in the same orchard (Tobin et al., 1996). Plotting individual locations by treatment week indicated that home range locations tended to shift slightly by week.

The mark–recapture results indicated that the proportion of captures in traps within or adjacent to treated trees tended to decrease over the treatment period on the TMT grids, but not significantly. This trend was also observed with the session 1 telemetry data (lower number of locations in treated areas), although statistical differences were not detected. However, the telemetry results from session 2 do not indicate any treatment differences. Therefore, our results do not support hypothesis 4 of a lower number of locations in trees treated with predator odors.

Throughout the entire study, none of the radio-collared rats traveled more than ~150 m from its original weekly home range location. This indicates that no rat ever left the grid on which it was originally trapped. The few rats that did travel greater than this distance either had their radio collar recovered (predation/slippage) or remained stationary underground, which is also likely to have resulted from predation. The live-trapping data also confirmed small home ranges, as no animal tagged on one grid was ever captured on any other grid. These results support similar findings showing that rats do not stray far from their home range (Spencer and Davis, 1950; Worth, 1950; Pippin, 1961; Tobin et al., 1996).

A potential explanation for this observation is the high abundance of year-round food coupled with a high density of individuals. Population density of roof rats has been associated with food availability in the Galapagos Islands (Clark, 1980), but this should not be a limiting factor in the orchard habitat. Rodents residing in the orchard have an almost continuous availability of nuts due to the prolonged flowering season and extended nut maturation period in Hawaii (Cavaletto, 1983). Studies have revealed that the roof rat's diet in orchards consists almost entirely of macadamia nuts, which allows rats to breed on a year-round basis (Tobin et al., 1993).

We found no differences in roof rat responses to DMDIT and TMT semiochemical treatments. A lack of response to the predator odor treatments may be a result of important habitat values present in the macadamia orchard: abundance of food, water, and cover. Other small mammal studies have demonstrated that cover is an important factor in the presence of predator odor (Merkens

et al., 1991). The lack of response may also be attributed to improper methodology to detect the response or low effectiveness of odor-release devices. Another potential explanation may be roof rat habituation to the odors or a lack of recognition of the semiochemicals, which were based on predator species not established in Hawaii. Although some studies indicate genetic recognition of odors (Gorman, 1984; Vernet-Maury et al., 1984, Boag and Mlotkiewicz, 1994), this theory is difficult to test, and perhaps learned behavior is more of a factor in this case. The roof rat's resilient nature probably allows for this adaptability, and many studies have reported its ability to learn new behaviors (Berdoy and MacDonald, 1991; Galef and Whiskin, 1994).

Methodology changes in future research into predator odor effects on roof rats should include a mark–recapture design with intensive sampling over a short period of time with longer intervals between sampling, and use of closed population analysis techniques. These closed population techniques have models that can allow for unequal capture probabilities, as occurred in this study. A more intensive sampling design (e.g., every two weeks) than used in our study may increase susceptibility to capture and allow use of open population analysis techniques.

Telemetry techniques should continue to be utilized as they provide specific information on individual movements. An important telemetry measurement to consider is that of shifts in center of activity. This could have been occurring in this study and may not have been properly addressed in the home range estimates and proportion of readings in treated trees.

For future studies, the importance of cover should also be explored through cover manipulation experiments coupled with mark–recapture and/or telemetry techniques. In addition, studies should focus on a population of rats that is well understood with respect to population parameters and movement patterns, with a treatment design maximizing the number of replicates. Whereas our results did not indicate semiochemical avoidance, recent findings with roof rat avoidance of mongoose feces in the field (Tobin et al., 1995) imply that potential responses may exist. Although semiochemicals from mongoose feces were not available at the time of this study, further research into the roof rat's response to this predator odor is recommended based on the laboratory findings of Burwash et al. (1998) and recent field results (Tobin et al., 1997).

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RESPONSE OF THE LACEWING *Chrysopa cognata* TO PHEROMONES OF ITS APHID PREY

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Abstract—The lacewing *Chrysopa cognata*, one of the principal predators of aphids in Korea, was tested for responses to the aphid sex pheromone components (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and the aphid alarm pheromone (*E*)-β-farnesene. Electroantennogram responses were obtained to the sex pheromone components but not to (*E*)-β-farnesene. The sex pheromone components were attractive in a Y-tube olfactometer assay and in field trials with water traps, but no attraction was observed to (*E*)-β-farnesene.

Key Words—Kairomone, *Chrysopa cognata* Wesmael, aphid pheromones, cyclopentanoids, nepetalactone, nepetalactol, (*E*)-β-farnesene, Y-tube, electroantennogram, field trapping.

INTRODUCTION

Aphids (Homoptera: Aphididae) employ various pheromones that mediate behavior, particularly mating and alarm responses (Pickett et al., 1992), and these pheromones play important roles in reproduction and survival against predation and parasitism. Many species of aphids reproduce sexually on their primary hosts during the autumn, and the sexual females (oviparae) produce a sex pheromone that attracts males (Pettersson, 1970, 1971). The sex pheromone for a number of aphid species, including the black bean aphid, *Aphis fabae* Scop., the pea aphid, *Acyrthosiphon pisum* (Harris), the greenbug, *Schizaphis graminum* (Rond.), and the peach-potato aphid, *Myzus persicae* (Sulz.), have been

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identified and shown to comprise one or both of (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Dawson et al., 1987, 1988, 1990, 1996; Pickett et al., 1992). It has been suggested that aphid sex pheromones may act as kairomones in attracting predators and parasitoids (Dawson et al., 1987). In a field trial investigating the response of male aphids to synthetic aphid sex pheromone, hymenopterous parasitoids, principally the braconid *Praon volucre* Haliday, were attracted to pheromone traps (Hardie et al., 1991). This finding suggested that such parasitoids may utilize aphid sex pheromone components in host location. However, to date, the attraction of predators to aphid sex pheromones has not been reported.

The aphid alarm pheromone is released, together with a sticky defense secretion, from the cornicles of aphids when they are attacked by predators or parasitoids. The main component of this pheromone for most species is the sesquiterpene hydrocarbon (*E*)-β-farnesene, which induces various types of defensive or avoidance behavior in aphids (Edwards et al., 1973; Pickett and Griffiths, 1980; Dawson et al., 1990; Nault and Phelan, 1984). It also has been suggested that the aphid alarm pheromone may attract predators or parasitoids, but this hypothesis has not been demonstrated (Nakamuta, 1991; Pickett et al., 1992). However, the biosynthetically related sesquiterpene hydrocarbon (−)-β-caryophyllene, a potent inhibitor of the aphid alarm pheromone (Dawson et al., 1984), is reported to attract the lacewing *Chrysoperla carnea* (Neuroptera: Chrysopidae) (Flint et al., 1979).

Lacewings are considered to be one of the most effective general entomophagous predators and are found in most of the agriculturally important areas of the world (New, 1988). Lacewings feed on eggs and young larvae of moths, aphids, mites and a wide range of adults and immatures of soft-bodied insects (New, 1988). Some species prefer aphids to other prey when offered a choice (Narvallee and Shaw, 1969). In Korea, *Chrysopa cognata* Wesmael is one of the commonest lacewings and since this species shows a preference for aphids, its use is being developed for aphid control. In this study, aphid pheromone components were investigated to determine their influence on behavior of *C. cognata* as a basis for exploiting their use in improving biological control of aphids.

METHODS AND MATERIALS

Insects. Larvae and adults of *C. cognata* were maintained at a temperature of 25°C, 50% ± 10% relative humidity, under a 16L:8D regime. Adults were kept in 30-cm acrylic cages and supplied with the aphid *M. persicae* as food. Eggs, harvested daily from the cages, were placed individually in plastic cups (3 cm diameter, 4 cm height), and aphids were added after the larvae hatched.

Cocoon formation and pupation occurred in the cups. Newly emerged adults were collected daily and transferred to acrylic cages for oviposition or, at 3 to 7 days old, were used for behavioral and electrophysiological studies. *M. persicae* were reared on radish seedlings (*Rhaphanus sativus* L.) under the same environmental conditions.

Chemicals. (4a*S*,7*S*,7a*R*)-Nepetalactone (98.1%), (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (98.4%) and (*E*)-β-farnesene (89.7%) were prepared by methods described previously (Pickett et al., 1992).

Olfactometer Studies. All experiments were conducted in a glass Y-tube olfactometer (stem 2 × 10 cm, each arm 2 × 12 cm). Air (1.2 liter/min) was drawn through the olfactometer by a vacuum pump connected to a flowmeter. Each arm led from a sample container that incorporated the source of test material. The arms were wrapped in white paper to exclude visual effects. Separation of the odor plumes at the junction of the two arms was confirmed with $TiCl_4$ smoke. Thirty replicates were employed and each olfactometer was used only three times in one series of assays. The olfactometers were rotated 180° after each replicate to exclude directional bias. At the end of a bioassay series, all olfactometers were washed with solvent in the following order: tap water, dilute anionic detergent, tap water, analytical grade methanol, and distilled water, and then heated overnight at 180°C.

The source of test material used in the bioassays was a borosilicate vial (08-CPV, Chromacol) containing the pheromone component in 50 μl ether and having a 1-mm-diameter hole drilled in the polyethylene cap. Individual lacewings were introduced into the stem of the Y-tube olfactometer and their positions noted 5 min after passing the start line, set arbitrarily at 5 cm from the base. Lacewings passing the final choice line (arbitrarily set at 8 cm from the arms' branching point) were recorded as positive and those remaining in the stem were recorded as no response.

To establish optimum conditions for the olfactometer studies, two aspects were initially examined: (1) the effect of the concentration of (4a*S*,7*S*,7a*R*)-nepetalactone on the response of lacewings, and (2) the periodicity in the response to (4a*S*,7*S*,7a*R*)-nepetalactone during the photophase. As a result, all bioassays were performed during 7–9 hr after commencement of the photophase.

Electrophysiology. Electroantennogram (EAG) responses of *C. cognata* were recorded with a capillary electrode filled with 3 M KCl solution and an indifferent electrode of electrolytically sharpened tungsten wire. EAG responses from the preparation were amplified 10 times (DAM70 amplifier) and the signals stored on an oscilloscope (Philips PM3350A). Permanent copies of the EAG responses were printed by a recorder (LKB 2210).

The legs and thorax of the lacewing were affixed to a glass slide with adhesive tape and the antenna was secured in position with a 2-mm strip of tape. The indifferent electrode was inserted into the abdomen and the recording

electrode was placed over the distal end of the antenna, from which the tip (approx. 2 mm) had been removed.

Purified air was passed continuously over the preparation through one arm of a bifurcated glass tube, the stem of which was positioned approx. 2 cm from the preparation. The stimulus was applied from a glass syringe via the other arm of the tube. Test samples in ether (50 μl) were applied to a filter paper disc and 1 min allowed for evaporation of the solvent; the disc was then placed in the syringe and a further 2 min allowed for equilibration. The syringe was connected to the second arm of the bifurcated tube, and a 1-ml puff of air containing the test material was delivered into the continuous air flow. Test samples were presented in the following order: diethyl ether, a test material, diethyl ether, etc., with 5 min intervals between stimulations.

Field Trapping Test. The aphid sex pheromone components (10 mg in 50 μl ether) were placed in individual borosilicate vials as used for the olfactometer assay. Under laboratory conditions at 20°C, these lures have been shown to release 200 μg/day over a five-week period (Gabryś et al., 1998). To obtain different release ratios, various numbers of nepetalactol and nepetalactone vials were placed in the water traps. To ensure similarity of visual cues, additional vials containing 50 μl ether were added so that all traps had five vials. Water traps were constructed from clear plastic Petri dishes (16 cm diameter; 2.5 cm deep), mounted 1.1 m above the ground and 1.5 m apart from each other. These were filled with a dilute anionic detergent solution and pheromone vials were mounted centrally, close to the liquid surface as described by Hardie et al. (1991).

Field trials were conducted at five sites in a peach orchard in Suwon, Korea from November 17 to December 20, 1994. Each site contained eight water traps, seven of which had pheromone lures releasing different proportions of nepetalactone and nepetalactol (ratios of 1:0, 4:1, 2:1, 1:1, 1:2, 1:4, 0:1) and one solvent control. Previous studies (Hardie et al., 1994) showed that relatively stable release ratios of the two components were maintained for two weeks. All lures used in the trials were therefore replaced at two-week intervals.

RESULTS

Olfactometer Studies. Before preferences to the aphid pheromone components were examined, the response to different doses of (4a*S*,7*S*,7a*R*)-nepetalactone was tested in the Y-tube olfactometer. The frequency with which lacewings chose one of the two arms of the Y-tube olfactometer was used as an index for the responsiveness to different doses. The lacewings responded in a dose-dependent manner to the nepetalactone (Table 1), with the highest level

TABLE 1. *C. cognata* RESPONDING IN Y-TUBE OLFACTOMETER CHOICE TESTS TO (4*aS*,7*S*,7*aR*)-NEPETALACTONE ($N = 30$)[a]

Amount of nepetalactone (mg)	*C. cognata* responding (N)		P	No response (N)
	Treated arm	Control arm		
0	7	6	NS	17
2	8	7	NS	15
4	17	2	<0.01	11[b]
6	14	6	NS	10[b]
8	24	2	<0.01	4[b]
10	26	4	<0.01	0[b]

[a]NS = no significant difference between treated and control arms (classical sign-test, $P < 0.05$).
[b]Significant difference from solvent control (classical sign-test, $P < 0.05$).

of response observed to the highest treatment (10 mg). Thereafter, all Y-tube olfactometer choice tests were performed with this dose.

The lacewings showed very low general locomotor activity at the beginning of the photophase but activity increased with time. To test whether this diel periodicity had a bearing on the response to aphid sex pheromones, Y-tube olfactometer choice tests were again performed with the 10-mg (4a*S*,7*S*,7a*R*)-nepetalactone stimulus. A clear circadian rhythm was apparent in the response to the test compound, with the highest response exhibited during the period 7–9 hr after the beginning of the photophase (Table 2). Thereafter, all Y-tube choice tests were performed during this period.

Both sexes of *C. cognata* were attracted to the aphid sex pheromone components (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol in the Y-tube olfactometer. There was no significant difference in response of male or female lacewings (Table 3). In a competitive assay, significantly more lacewings were attracted to the 10-mg stimulus of the nepetalactone than to a similar concentration of the nepetalactol (Table 4). No attraction was observed in the olfactometer to 10 mg of the alarm pheromone (*E*)-β-farnesene; however, the number of individuals showing no response was significantly lower than in the control bioassay (Table 4).

Electrophysiology. For *C. cognata*, the amplitude of the EAG response decreased throughout the life-span of the preparation, even though the preparation usually lasted for 5–6 hr (Figure 1). However, the ratio of responses to the compounds remained almost constant. Consequently, all EAG responses were normalized to give equivalent initial amplitudes, expressed in millivolts,

TABLE 2. *C. cognata* RESPONDING IN Y-TUBE OLFACTOMETER CHOICE TESTS TO (4*aS*,7*S*,7*aR*)-NEPETALACTONE (10 mg) AT DIFFERENT TIMES DURING PHOTOPHASE ($N = 30$)[a]

Hours after start of photophase	*C. cognata* responding (N)		No response (N)
	Treated arm	Control arm	
1	3c	4	23a
3	12b	3	15ab
5	16ab	7	7bd
7	27a	3	0c
9	18ab	12	0c
11	22ab	5	3cd
13	20ab	5	5d
15	18ab	7	5d

[a]Numbers followed by the same letter in the columns are not significantly different (χ^2 test, $P < 0.05$).

against the mean response for the solvent, diethyl ether, applied before and after the test material. EAGs were recorded at least at 5-min intervals (Figure 2).

EAG responses of *C. cognata* to (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol increased in a typical dose-dependent manner, with

TABLE 3. MALE AND FEMALE *C. cognata* RESPONDING IN Y-TUBE OLFACTOMETER CHOICE TESTS TO TEST MATERIALS (10 mg) ($N = 30$)[a]

Stimulus	Sex	*C. cognata* responding (N)		No response
		Treated arm	Control arm	
Nepetalactone	Male	24a[b]	6b	0c
	Female	22a[b]	8b	0c
Nepetalactol	Male	19a[b]	8b	3c
	Female	20a[b]	9b	1c

[a]Numbers followed by the same letter in the columns are not significantly different (χ^2 test, $P < 0.05$).
[b]Significant difference between treated and control arms (classical sign-test, $P < 0.05$).

TABLE 4. RESPONSE FREQUENCY OF *C. cognata* IN Y-TUBE OLFACTOMETER CHOICE TESTS TO TEST MATERIALS (10 mg) ($N = 30$)[a]

	Response (N)			No response (N)[b]
	Arm 1	Arm 2	P	
Empty vs. empty	5	7	NS	18a
Nepetalactone vs. nepetalactol	22	8	<0.05	0b
EBF vs. empty	11	13	NS	6c

[a]Empty: solvent (diethyl ether) only. EBF: (*E*)-β-farnesene. NS = no significant difference between arms 1 and 2 (classical sign-test, $P < 0.05$).
[b]Numbers followed by the same letter are not significantly different (χ^2 test, $P < 0.05$).

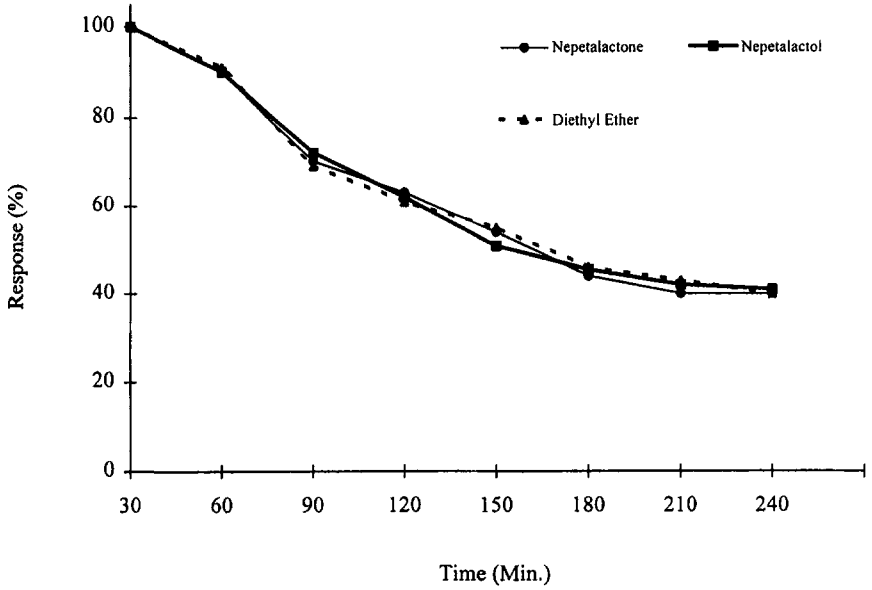

FIG. 1. Decrease with time in the EAG response of *C. cognata* to test materials (50 μg) in comparison to initial response amplitude ($N = 1$).

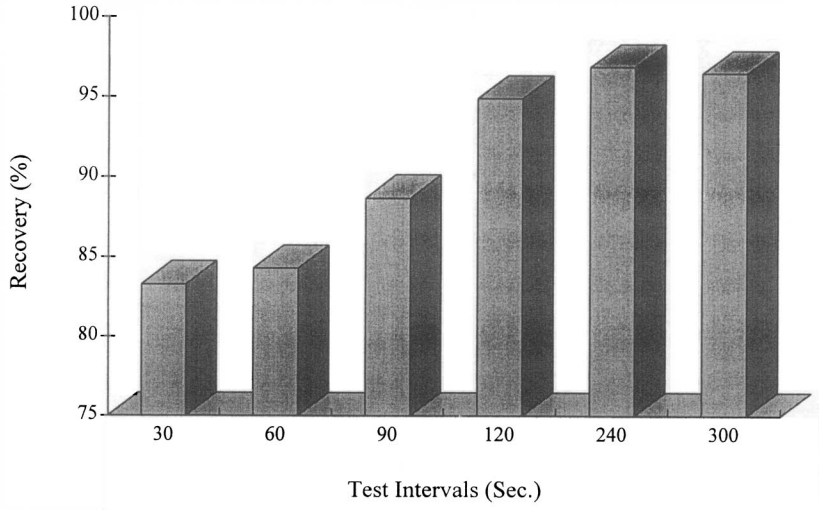

FIG. 2. Recovery with time in the EAG response of *C. cognata* to the nepetalactone (50 μg). The EAG response is expressed as a percentage of the initial amplitude ($N = 1$).

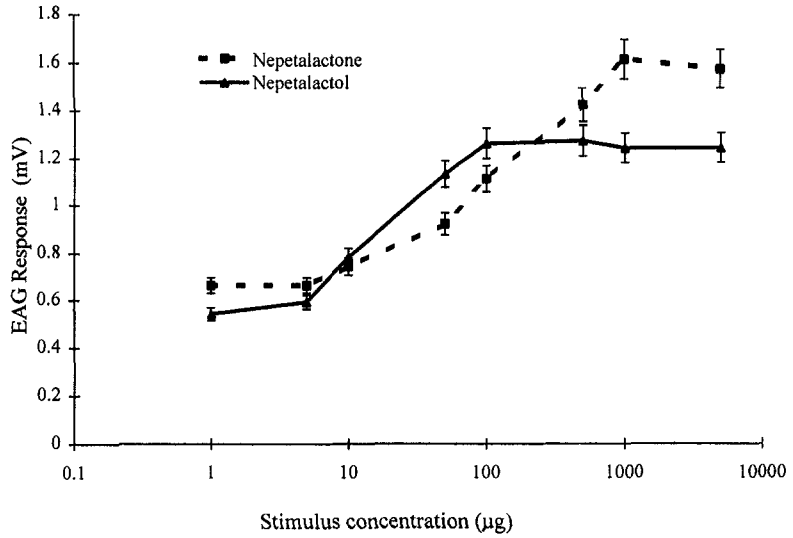

FIG. 3. Normalized EAG response of *C. cognata* to various amounts of the nepetalactone and the nepetalactol ($N = 3$).

TABLE 5. NORMALIZED EAG RESPONSES OF FEMALE *C. cognata* TO TEST MATERIALS (50 μg) ($N = 1$)

Stimulus	Peak value (mV)	Rising rate (mV/sec)	Returning rate (mV/sec)	Response time (sec)
Air (control)	0.24	2.4	2.4	0.2
Diethyl ether	0.24	2.4	2.4	0.2
EBF[a]	0.24	2.4	2.4	0.2
Nepetalactone	1.61	15.2	1.0	1.2
Nepetalactol	1.22	12.2	1.4	0.8

[a]EBF: (*E*)-β-farnesene.

similar threshold stimulus concentrations for both components (Figure 3). However, saturation of the EAG dose-response curve for the nepetalactol was observed at the 100 μg stimulus level, whereas saturation concentration for the nepetalactone was 1000 μg. No EAG response was elicited by (*E*)-β-farnesene (Table 5), even when the amount was increased to 10 000 μg (data not shown).

Field Trapping. Over the autumn 1994 trapping period (29 days), a total of 260 *C. cognata* were caught in water traps treated with the sex pheromone components, whereas only one individual was caught in the control traps. Although only the 1:4 and 4:1 mixtures of (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol attracted significantly more lacewings than the individual components, all pheromone-treated traps caught many more than the solvent control (Figure 4). When the numbers of lacewings caught with the single-component lures were compared, the (4a*S*,7*S*,7a*R*)-nepetalactone attracted over three times as many as the (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Figure 4).

DISCUSSION

This study indicates that aphid sex pheromone components are potent attractants for the lacewing *C. cognata*, one of the principal predators of aphids in the Far East. This is the first evidence for the employment of aphid sex pheromones as kairomones by an aphid predator.

The use of sex pheromones as kairomones for host location has been reported in several moth egg parasitoids (Noldus and van Lenteren, 1985; Nordlund et al., 1983), where such stimuli might provide cues for location of host oviposition sites, either when the female lays eggs close to the sex pheromone release site or if she leaves traces of sex pheromone at the oviposition site. Female noctuid

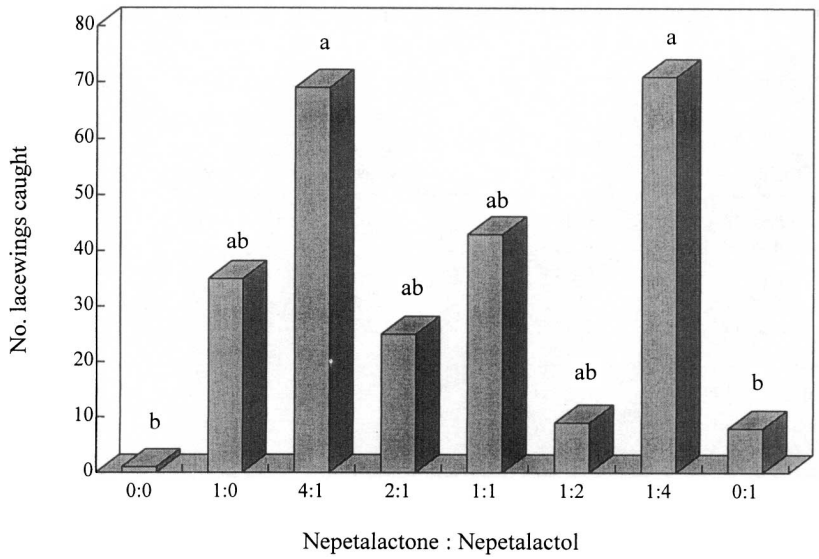

FIG. 4. Total numbers of *C. cognata* caught in water traps treated with aphid sex pheromone components and a solvent control in autumn 1994. Columns with the same letter are not significantly different at $\alpha = 5\%$ (Duncan's NMRT).

moths releasing pheromone in the presence of host plants usually lay eggs nearby. Absorption and rerelease of pheromone from leaves could provide a relevant cue for the location of eggs (Noldus et al., 1991; Stowe et al., 1995). Attraction of parasitoids to sex pheromones of adult hosts also has been reported (Mitchell and Mau, 1971; Abdel-Kariem and Kozar, 1988).

Stowe et al. (1995) suggested that predators in the Arthropoda are able to intercept sex pheromone signals of their prey, but they presented no examples of predatory insects' attraction to a prey sex pheromone source. Although there are many reports on the employment of host sex pheromones by parasitoids, Wood (1982) listed only a few predators of bark beetles utilizing sex pheromones as a kairomone of their hosts. Zegelman et al. (1993) collected large numbers of male and female *Elatophilus herbraicus* (Hemiptera: Anthocoridae), an obligatory predator of the Israel pine bast scale *Matsucoccus josephi* (Homoptera: Matsucoccidae), by means of the host sex pheromone. This current study adds to this list another predator being attracted to the sex pheromone of its prey. However, this is the first demonstration of a predator of an agricultural pest, and certainly a predator of aphids, locating its prey by means of a sex pheromone.

Members of the Chrysopidae (green lacewings) are predominantly polyphagous predators and accept aphids as a part of a broad spectrum of soft-bodied and slow-moving prey in the Arthropoda (New, 1988). It is therefore somewhat unexpected that such a general predator as *C. cognata* utilizes the aphid sex pheromone as a kairomone. It was suggested that general predators might respond to more common biochemicals, such as amino acids, whereas highly specialized predators, such as those attacking scolytid beetles, may respond to semiochemicals more closely associated with their hosts or the host plant complex (Greany and Hagen, 1981). However, the fact that a predator is relatively polyphagous does not preclude its use of a specific chemical cue for prey finding and, indeed, there are reports that general feeders are also attracted to odors from their prey (Greany and Hagen, 1981).

The response of *C. cognata* to aphid sex pheromones appears analogous to that observed for the generalist aphid parasitoids in the genus *Praon* (Hymenoptera: Braconidae). For parasitoids, only the females need to locate aphids for oviposition, whereas for predators, both sexes usually feed on a prey species. Although EAG responses were obtained from *Praon* spp. females to both aphid sex pheromone components (Hardie et al., 1993; C. M. Woodcock, unpublished), trap catches in the field were particularly associated with the presence of the nepetalactone (Hardie et al., 1991, 1994). Similarly, in this study, the nepetalactone showed higher kairomonal activity than the nepetalactol, suggesting that, since this compound is a more common sex pheromone component, the more general aphid signal is predominantly being employed (Dawson et al., 1996).

An unexpected finding in this study is the lack of both EAG and behavioral responses of *C. cognata* to the aphid alarm pheromone (*E*)-β-farnesene. These electrophysiological results contrast with those observed for the lacewing *Chrysoperla carnea*, where (*E*)-β-farnesene elicited an EAG response about 30% higher than either of the two sex pheromone components (C. M. Woodcock, unpublished). However, the lack of an EAG response here may relate to a low proportion of olfactory receptors for this compound on the antenna (Wadhams, 1990). It should be noted that while no significant attraction of *C. cognata* to (*E*)-β-farnesene was observed in the olfactometer tests, the number of individuals showing no response, i.e., no movement, was significantly lower than in the control bioassay (Table 4), suggesting that the alarm pheromone may have some role in the chemical ecology of this insect. Nevertheless, it is somewhat surprising that *C. cognata* was attracted, both in the olfactometer and in the field, to the aphid sex pheromone, which is released over a limited period in the late autumn, and yet showed no response to the alarm pheromone, which is released throughout the season, whenever aphids are disturbed or, more particularly, attacked. Thus, although Nakamuta (1991) suggested that the aphid alarm

pheromone may act as a kairomone for predators, this hypothesis has not yet been proven for *C. cognata*.

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PHYTOTOXIC IRIDOID GLUCOSIDES FROM THE ROOTS OF *Verbascum thapsus*

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Abstract—The iridoid glucosides lateroside **1**, harpagoside **2**, ajugol **3**, and aucubin **4** were isolated from an ethanolic extract of the roots of the weed *Verbascum thapsus* that exhibits antigermination activity on seeds of barley (*Hordeum vulgare*). Bioassays indicated that at 3 mM concentration, compounds **1**, **2**, and **4** showed moderate inhibition of seed germination. These compounds also reduced root length when they were assayed on pregerminated seeds at 1 mM to 0.001 mM concentration range. Of all compounds tested, aucubin **4** was the most active against root elongation. Compound **3** showed no activity in the bioassays.

Key Words—*Verbascum thapsus*, weeds, iridoid glycosides, phytotoxicity, barley, *Hordeum vulgare*, germination, root inhibition.

INTRODUCTION

Weed species are frequently considered to be competitive because they show vigorous growth in crops and reduce crop yield. It is known that the possession of certain biological characteristics has the potential to predispose a species to exhibiting weediness. Regarding these characteristics, an ideal weed would show strong interspecific competition via special mechanisms such as allelopathic

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processes (Mortimer, 1990). Thus, allelopathic chemicals may play an important role in determining the persistence and abundance of weed species in mixtures of plants.

The allelopathic effects of weeds on crop plants has been intensively studied since 1970 (Rice, 1979). According to Rice, the modification of seed germination and plant growth is one of the more obvious manifestations of allelopathy, and a germination bioassay is an important tool for studying this phenomenon.

In order to study chemical plant–plant interactions, we assayed polar extracts from some weed species growing in southern Chile (Espinoza, 1988) for antigermination activity on barley seeds. In this screening the crude ethanolic extract of fresh roots of *Verbascum thapsus* L. (Scrophulariaceae), a weed indigenous in Europe that also is widely distributed in southern Chile, showed strong inhibition of barley germination. Previous investigation on the whole plant of *V. thapsus* described the isolation of verbascoside (Mehrotra et al., 1989), iridoid glycosides (Seifert et al., 1985; Khuroo et al., 1988; Warashina et al., 1991), sterones and a sesquiterpene acid (Khuroo et al., 1988), and phenylethanoid and lignan glycosides (Warashina et al., 1992). Although the composition of *V. thapsus* has been extensively studied, there appear to be no reports in the literature of the plant being fractionated for antigermination activity. Thus, a question remains as to which chemical substances contained in *V. thapsus* exert antigermination activity. Therefore, it was intended in this study to identify phytotoxins from *V. thapsus* roots by using chemical methods and to investigate their effects on barley seed germination and growth.

METHODS AND MATERIALS

Screening for Antigermination in Weeds. Ten weed species habitually growing in southern Chile (Espinoza, 1988)—*Sylybum marianum* (L.) Gaerth. (milk thistle, Asteraceae), *Cirsium vulgare* (Savi) Ten. (bull thistle, Asteraceae), *Cichorium intybus* L. (chicory, Asteraceae), *Centaurea melitensis* L. (tocalote, Asteraceae), *Rumex crispus* L. (curly dock, Polygonaceae), *Rumex acetocella* L. (red sorrell, Polygonaceae), *Rumex pulcher* L. (fiddle dock, Polygonaceae), *Ulex europaeus* L. (gorse, Fabaceae), *Verbascum thapsus* L. (common mullein, Scrophulariaceae), and *Echium vulgare* L. (blueweed, Boraginaceae)—were collected in the wild.

Fresh root samples of each plant were macerated with 96% ethanol and filtered. After evaporation under vacuum, the resulting crude extracts were tested for activity on barley according to the germination bioassay described below.

Extraction and Preliminary Biological Evaluation of V. thapsus *Roots.* The roots of *V. thapsus* were collected in Cajón, Temuco, in December 1994; a voucher specimen was deposited in the Department of Chemical Sciences, Uni-

versidad de La Frontera, Temuco. Approximately 3 kg of *V. thapsus* fresh roots were extracted with 7 liters of 96% ethanol for three days. The volume of the extract was reduced on a rotatory evaporator, and yielded about 20 g residue. The resulting crude extract (20 g) was applied to a silica gel (Merck 60, 70–230 mesh) flash column and components on the column were sequentially eluted with 1 liters of chloroform, ethyl acetate, acetone, and ethanol. The ethyl acetate and acetone fractions were active in bioassays for antigermination of barley seeds.

Isolation and Identification of Active Compounds. The active acetone fraction (7 g) was further separated by silica gel (Merck 60, 230–400 mesh) column chromatography. The column was washed with increasing volumes of MeOH in $CHCl_3$ (0–40% v/v), and 25 fractions (70 ml) were collected. All fractions obtained from the column were bioassayed for activity on barley and qualitatively evaluated by silica gel TLC (Merck 60 F_{254}). Zones were visualized under UV light and by spraying with AcOH–H_2O–H_2SO_4 (80:16:4) solution and then heating at 120°C. The active fractions from 10% MeOH were combined and evaporated to dryness, and the residue (180 mg) was further purified on silica gel preparative TLC (Merck 60 F_{254} precoated plates). The developing solvent was C_6H_6–EtOH (3:1). Compounds **1** (30 mg) and **2** (30 mg) were isolated. The active fractions eluting with 20% MeOH were combined to yield a 220 mg sample. Further purification on silica gel preparative TLC ($CHCl_3$–MeOH) (3:1) afforded, in order of increasing polarity, compounds **3** (15 mg) and **4** (65 mg). The pure compounds were identified by ^1H and ^{13}C NMR spectral data analysis and by comparison with data previously reported [**1**, **3**: Swiatek et al. (1981); **2**: Sticher and Afifi-Yazar (1979), **4**: Chaudhuri et al. (1980) and Swiatek et al. (1981)]. NMR spectra for compounds **1–4** were recorded in CD_3OD on a Varian XL-300 spectrometer, operating at 300 MHz for ^1H and 75 MHz for ^{13}C nuclei. The chemical shifts are reported in δ (ppm), with TMS as an internal standard. Multiplicity and coupling constants (J/Hz) are given in parenthesis.

Germination Bioassay. Filter paper germination bioassays were conducted as described by Wolf et al. (1984). Thus, known sample weights were applied in 0.5 ml MeOH to Whatman No. 1 filter paper in Petri dishes (90 × 15 mm). Pure solvent and water were similarly applied to filter paper as separate controls. After solvent evaporation, 4 ml of water were added to all dishes. Ten barley seeds (99% germination capacity) were added and dishes were incubated for four days in a high humidity growth chamber (27°C, 16 hr daylight), and the number of germinated seeds was recorded. The effect of the pure compounds was determined at 3 mM concentration, based on earlier published work (Cameron et al., 1984; Wolf, 1986). For crude extracts, 40 mg of material were applied per dish, according to Miller et al. (1988). Each determination was replicated five times.

Root Growth Bioassay. In order to evaluate quantitatively the effect of phytotoxic substances on the root growth, experiments were conducted with barley seeds 24 hr after their germination in water. Seeds were considered germinated when the protrusion of the radicle became evident. In the bioassay, 0.1–0.001 mM solutions of substances **1**, **2**, and **4** were obtained from a methanolic stock solution (1.0 mM). Aliquots of each solution were adsorbed by filter paper discs. After solvent evaporation, pregerminated seeds were placed on treated filter paper in Petri dishes (96 × 15 mm), 10 per dish, and five dishes per treatment. To each dish, 4 ml of water was added, and the dishes were incubated in a high humidity growth chamber at 27°C with 16 hr daylight. Effects on root elongation were determined by measuring to the nearest millimeter the length of the radicle of each seedling six days after the start of the experiment (Aliotta et al., 1993). For every five treatments, a blank control was added. Values of 50% inhibition of root growth (I_{50}) related to control growth (100%) were calculated by probit analysis (Scott et al., 1984) from dose–response bioassays.

RESULTS

Initial screening of 10 species for antigermination, in order to identify species for further study, indicated that all 10 species showed some degree of activity on barley germination compared with the control. Especially severe inhibitory effects were observed in germination of barley seeds on filter paper treated with *R. crispus*, *V. thapsus*, and *E. vulgare* extract solutions (Table 1).

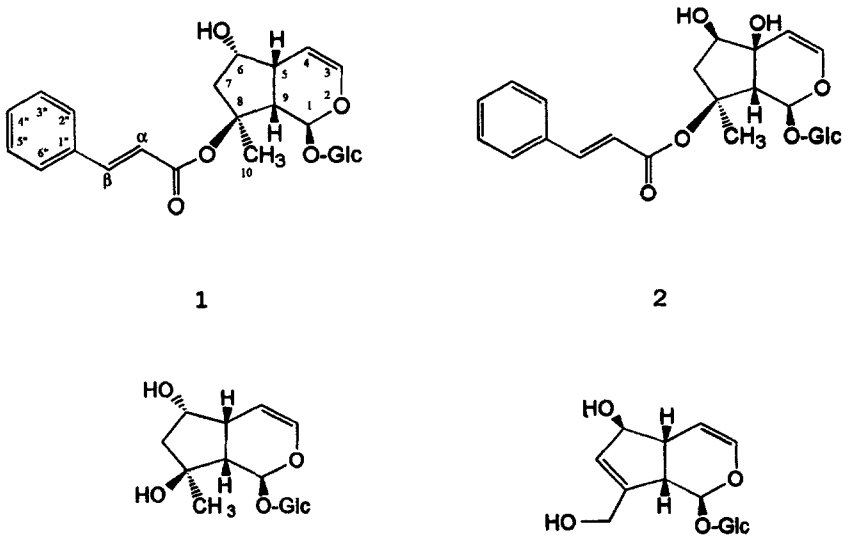
TABLE 1. EFFECTS OF DIFFERENT WEED SPECIES EXTRACTS ON BARLEY SEED GERMINATION

Species	Germination (% of control)[a]
Silybum marianum	65
Cirsium vulgare	77
Cichorium intybus	95
Centaurea melitensis	88
Rumex crispus	29
Rumex acetocella	63
Rumex pulcher	70
Ulex europaeus	52
Verbascum thapsus	19
Echium vulgare	30

[a] Data refer to both MeOH and water controls.

From earlier studies on the whole plant of *V. thapsus* and the results in the initial screening of species, we chose the weed *V. thapsus* for further study. According to the preliminary biological evaluation of *V. thapsus* ethanol extracts, the greatest antigermination activity was observed in the ethyl acetate and acetone fractions obtained from silica gel flash column chromatography. We chose in this study the most polar active fraction, the acetone-eluted fraction, for subsequent isolation work. Using bioassay-guided fractionation and purification, we isolated and identified the iridoid glucosides **1–4** as the major constituents of this fraction (Figure 1). The NMR data we obtained from the compounds are as follows:

Lateroside 1. Proton (CD_3OD): 7.66 (H-α, d, 16), 7.59 (H-2″, H-6″, m), 7.40 (H-3″, H-4″, H-5″, m), 6.50 (H-β, d, 16), 6.23 (H-3, dd, 6.3; 2.3), 5.97 (H-1, 5), 4.73 (H-4, dt), 4.68 (H-1′, d, 7.9), 4.06 (H-6, d, 4.6), 3.92 (H-6′a, dd, 12; 2), 3.67 (H-6′b + H-4′, m), 3.37 (H-3′, d, 9), 3.30 (H-5′ + CD_3OD), 3.20 (H-2′, dd, 7.9; 9), 2.95 (H-9, d, 8.4), 2.84 (H-5, m), 2.29 (H-7a, d, 14.9), 2.15 (H-7b, dd, 14.9; 4.6), 1.60 (Me-10, s).



1 2

3 4

FIG. 1. Iridoid glucosides from *V. thapsus* tested for phytotoxicity on barley germination and root growth.

Carbon (CD_3OD): 168.7 (CO), 145, 9 (C-α), 141.6 (C-3), 135.8 (C-1″), 130.0 (C-2″, C-6″), 131.7 (C-4″), 129.2 (C-3″, C-5″), 120.3 (C-β), 104.2 (C-4), 100.1 (C-1′), 94.7 (C-1), 90.3 (C-8), 78.2, 78.1 (C-3′, C-5′), 77.0 (C-6), 74.9 (C-2′), 71.7 (C-4′), 63.1 (C-6′), 49.6 (C-9), 48.9 (C-7), 41.8 (C-5), 23.0 (C-10).

Harpagoside **2.** Proton (CD_3OD): 7.67 (H-α, d, 16), 7.60 (H-2″, H-6″, m), 7.40 (H-3″, H-4″, H-5″, m), 6.52 (H-β, d, 16), 6.41 (H-3, d, 6.4), 6.18 (H-1, s), 4.90 (H-4, H-6, m) 4.61 (H-1″, d, 7.9), 3.92 (H-6′a), 3.75 (H-6′b, H-4′), 3.40-3.20 (H-3′, H-5′, H-2′ + CD_3OD), 2.93 (H-9, s), 2.27 (H-7a, d, 15), 2.02 (H-7b, dd, 15; 4.5), 1.53 (Me-10).

Carbon (CD_3OD): 168.7 (CO), 146.1 (C-α), 143.9 (C-3), 135.8 (C-1″), 131.5 (C-4″), 130.0 (C-2″, C-6″), 129.2 (C-3″, C-5″), 120.1 (C-β), 106.9 (C-4), 100.0 (C-1′), 94.6 (C-1), 88.8 (C-8), 78.2 (C-2′), 77.7 (C-3′), 77.6 (C-5′), 74.6 (C-6), 73.4 (C-5), 71.8 (C-4′), 63.0 (C-6′), 55.6 (C-9), 46.2 (C-7), 22.6 (C-10).

Ajugol **3.** Proton (CD_3OD): 6.15 (H-3, dd, 6.3; 2.1), 5.46 (H-1, d, 2.2), 4.85 (H-4, dd, 6.3; 2.8), 4.63 (H-1′, d, 7.9), 3.91 (H-6, H-6′a, m), 3.65 (H-6′b, dd, 11.9; 5.6), 3.40-3.20 (H-2′, H-3′, H-4′, H-5′, m), 2.72 (H-5, dd, 9.6; 2.5), 2.54 (H-9, dd, 9.6; 2.2), 2.04 (H-7a, dd, 13.4; 5.6), 1.79 (H-7b, dd, 13.4; 5.6), 1.31 (Me-10).

Carbon (CD_3OD): 140.4 (C-3), 105.9 (C-4), 99.4 (C-1′), 93.7 (C-1), 79.4 (C-8), 78.2 (C-6), 78.0 (C-3′), 77.7 (C-5′), 74.8 (C-2′), 71.7 (C-4′), 62.8 (C-6′), 51.8 (C-9), 50.0 (C-7), 41.2 (C-5), 25.2 (C-10).

Aucubin **4.** Proton (CD_3OD): 6.30 (H-3, dd, 6.1; 2.0), 5.76 (H-7, br q), 5.09 (H-4, dd, 6.1; 3.9), 4.95 (H-1, d, 7.4), 4.67 (H-1′, d, 7.8), 4.42 (H-6, m), 4.34 (H-10a, dt, 16), 4.16 (H-10b, dt, 16), 3.85 (H-6′a, dd, 12.2; 1.7), 3.63 (H-6′b, dd, 12.2; 5.4), 3.40–3.18 (H-2′, H-3′, H-4′, H-5′, m), 2.88 (H-9, t, 7.4), 2.63 (H-5, m).

Carbon (CD_3OD): 148.0 (C-8), 141.6 (C-3), 130.2 (C-7), 105.7 (C-4), 99.9 (C-1′), 97.7 (C-1), 82.8 (C-6), 78.2 (C-3′), 77.8 (C-5′), 74.8 (C-2′), 71.5 (C-4′), 62.6 (C-6′), 61.4 (C-10), 47.9 (C-9), 46.3 (C-5).

We chose to test the compounds isolated from *V. thapsus* for antigermination effects at 3 mM concentration because of earlier work in which slightly water-soluble compounds related to *p*-metoxycinnamaldehyde (Wolf, 1986) and iridoids (Cameron et al., 1984) were bioassayed at 1–5 mM concentration in tests for antigermination or other effects on embryo growth activity. Our results are presented in Table 2.

Compounds **1** and **4** at 3 mM concentration showed moderate inhibition of barley germination (70% of the control). Compound **2** was the most active of all the compounds in the bioassays, allowing only 40% of the seeds to germinate. No inhibition of germination was observed when compound **3** was assayed at the 3 mM concentration.

TABLE 2. ACTIVITY OF IRIDOID GLUCOSIDES (3 mM) ON BARLEY SEED GERMINATION

Compound	Germination (% of control)[a]
Lateroside **1**	70
Harpagoside **2**	40
Ajugol **3**	100
Aucubin **4**	70

[a]Data refer to both MeOH and water controls.

TABLE 3. ACTIVITY OF IRIDOID GLUCOSIDES ON BARLEY ROOT ELONGATION

	Root length (% of control), concentration (mM)				I_{50}	
Compound	1.0	0.1	0.01	0.001	mM	mg
Lateroside **1**	54	60	75	80	2.3	1.10
Harpagoside **2**	51	62	68	77	1.7	0.84
Aucubin **4**	30	61	72	80	0.2	0.07

Pure compounds that had previously shown reduction in germination were subsequently assayed for activity on root elongation at concentrations ranging from 0.001 mM to 1.0 mM in a bioassay with barley seeds 24 hr after their germination in water (Table 3). All compounds tested exhibited some degree of activity in dose–response bioassays. Increasing concentrations of compounds reduced root length. Compound **1**, like **2**, showed moderate inhibition effect on root growth. The I_{50} values for root inhibition in response to **1** and **2** were 2.3 mM (1.1 mg) and 1.7 mM (0.84 mg), respectively. Compound **4** had the greatest activity with an I_{50} of 0.07 mg or 0.2 mM.

Although compound **2** had the highest activity on barley germination, compound **4** was, by far, the most active chemical affecting root growth.

DISCUSSION

Our findings agree well with those reported by Dornbos and Spencer (1990), who showed that phytotoxins producing the largest reductions in germination percentage did not necessarily cause the largest reductions in seedling length.

Aucubin **4** was the most potent root growth inhibitor and was present in high concentrations in the plant. Bioassays of all compounds tested showed no significant effects on barley shoot growth.

Harborne (1989, 1993) reviewed the role and importance of some iridoids as plant toxins that are sequestered by insects from food plants and stored for defense. Some iridoids, such as plumieride and their aglycone plumieridine, also have been reported as plant growth inhibitors on wheat seedling (Adam et al., 1979; Schliemann and Adam, 1982). Geniposide and geniposidic acid together with their aglucones inhibit the growth of wheat embryos (Cameron et al., 1984). Two iridoid glucosides related to asperuloside seem to be responsible for growth and germination inhibition of lettuce (Komai et al., 1986). In addition, geniposide and genipin gentobioside inhibit the germination of *Gardenia jasminoides* and watercress seeds and the growth of Chinese cabbage roots; the aglycone genipin also shows a strong effect on the growth of Chinese cabbage roots (Shimomura et al., 1983). Our results represent the first report of compounds **1**, **2**, and **4** exhibiting antigermination activity on barley seeds.

Some authors in preceding papers have discussed the correlation between the structure of certain iridoid glycosides and capacity to exert plant growth inhibitory activity. Thus, Adam et al. (1979) and Schliemann and Adam (1982) attributed the observed inhibiting activity of plumieride and its aglycone plumieridine to the presence of an α,β-unsaturated lactone group. However, no such group is present in the structure of the active iridoids geniposide and geniposidic acid, and Cameron et al. (1984) correlated activity with the iridoid structure itself. In our experiments, bioassays of compound **3** showed no inhibition of germination of 3 mM concentration. Thus, no clear pattern of germination inhibition related to compound structure could be established in the present series of iridoids.

In conclusion, the iridoid glucosides lateroside **1**, harpagoside **2**, and aucubin **4** appear to be responsible for the phytotoxicity of the root extracts of *V. thapsus* on barley seed germination and root growth. Further studies on the less polar active ethyl acetate fraction of the ethanol extract are in progress in our laboratory.

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TRANSFER, PERCEPTION, AND ACTIVITY OF MALE PHEROMONE OF *Acrolepiopsis assectella* WITH SPECIAL REFERENCE TO CONSPECIFIC MALE SEXUAL INHIBITION

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Abstract—In the leek moth, *Acrolepiopsis assectella*, the male, stimulated by a calling female, produces a sexual pheromone that is active on the female. The male-produced pheromone blend contains eight alkanes previously isolated from the male hair-pencils. We used EAG techniques to study the effect of the pheromone on females reared on leek or on artificial diet and on males reared on leek. The optimal stimulation duration appeared to be 1.6 sec exposure to the pheromone or components. The concentrations tested on the antenna were of the order of 10^{12} molecules/cm^3. The hair-pencil extract tested was of an estimated concentration of around 10^9 molecules/cm^3. The antennal responses are expressed relative to responses to a standard, amyl acetate, but also as an absolute value. Generally, females reared on artificial diet and males reared on leek responded better than females reared on leek. Of the alkanes tested, those present in hair-pencils gave higher responses, with hexadecane always giving the strongest response. A possible inhibiting activity of male leek moth pheromone on the sexual behavior of conspecific males was investigated. The behavior of sexually stimulated males was observed in the presence of other males, hair-pencil extracts, and different compounds either pure or in a mixture. The experiments established that in this species, male pheromone inhibits wing fluttering duration of conspecific males. This inhibition was obtained not only with fluttering males as a source of pheromone but also with all the alkanes tested. The inhibition was due to hair-pencil chemicals, particularly if these were perceived by olfaction plus contact. Wing fluttering increased the inhibitory activity of male-derived alkanes.

Key Words—Male pheromone, conspecific male, sexual inhibition, leek moth, EAG, alkanes, wing fluttering, hair pencils.

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INTRODUCTION

Mating in insects requires a preliminary exchange of information between adults of different sexes usually belonging to the same species. In numerous Lepidoptera, in which there is the emission of a pheromone signal from one sex, the opposite sex responds by producing physical or chemical signals. In the majority of cases studied, it is the calling female that attracts and stimulates the male, but males may also emit sexual pheromones.

Although emission, perception, and role of female pheromones often have been studied, male response is less well known, and the emission, perception, and role of male sex pheromones have been studied in only a limited number of species (Birch, 1974).

In the leek moth, *Acrolepiopsis assectella* (Zell.) (Lepidoptera: Hyponomeutoidea), a specialist phytophagous insect of *Allium* plants, males produce a sexual pheromone from the hair-pencils in response to female-produced sex pheromone (Thibout, 1972a). The pheromone plays an aphrodisiac role (Thibout, 1978) and has an arresting effect (Thibout et al., 1994) on calling females. The pheromone isolated from hair-pencils of *A. assectella* is composed of eight *n*-alkanes, ranging in size from hexadecane (C_{16}) to tricosane (C_{23}) (Auger and Ferary, 1994). These alkanes are present in very low quantities in males less than 24 hr old and their production seems to be independent of the larval host plant (Thibout and Auger, 1995). Extracts of hair-pencils taken from 4- to 6-day-old males that had fed as larvae on leek, *Allium porrum*, or on an artificial diet with or without leek powder contained very similar pheromonal components qualitatively and quantitatively.

While these alkanes are known to have an effect on female behavior (Thibout et al., 1994), they also may affect the behavior of males of the same species. In this study our objective was to determine with electrophysiology techniques whether these compounds are perceived by olfaction at the antennal level, and also to determine whether they have an effect on males. To determine the latter, we observed the behavior of sexually stimulated males in the presence of male pheromone.

METHODS AND MATERIALS

Insects. Leek moths were reared in the laboratory on *Allium porrum* in 16L:8D, at 25°C during the photophase and 16°C during the scotophase, and with 60–80% relative humidity. Males and females were separated after cocoon spinning, and the adults were kept in the presence of a small ball of damp cotton wool. The stock is renewed every year in July by harvesting larvae in leek fields

around Tours, France. Some of the EAG studies were conducted with adults obtained from a rearing made on an artificial diet containing freeze-dried leek powder (Arnault, 1982). This rearing procedure has been maintained for about 12 years at 14L:10D, 25°C, and 70% constant relative humidity. The leek moth is a nocturnal species, and sexual behavior occurs in the second half of the scotophase (Thibout, 1974). Thus, the adults used for behavioral experiments were held at 25°C constant temperature in a reversed photoperiod (scotophase from 04:00 hr to 12:00 hr). Light of 0.6 lux enabled observation of individuals without disturbing them.

Sensory Perception. Sensory perception was analyzed by EAG during the scotophase with adults 4 to 7 days old. Three groups of adults were used in EAG experiments: males reared on leek, females reared on leek, and females reared on artificial diet with added leek powder. The EAG technique has been used with this species previously (Renou et al., 1981; Lecomte and Pouzat, 1986), and it has been shown that olfactory perception of female pheromone and plant odors takes place on the antennae.

The materials and the technique for EAG have been described previously for other species (Pouzat and Nammour, 1989; Chaibou et al., 1993). Only the air source for stimulus delivery was modified so that the antenna, which was permanently subjected to 30 cm/sec airflow, showed no response to the control airflow. Stimulation durations of 0.8, 1.6, 3.2, and 6.4 sec were studied. Five to eight odors were tested in varying order with an antenna. A standard stimulation with 4×10^{-2} mol/liter amyl acetate was interspersed after every two stimulations with a test odor. There were two puffs with pure air between each stimulation. A preliminary experiment carried out on about 10 adults of each experimental group with C_{16}, C_{17}, C_{19}, and C_{23} alkanes enabled the optimum duration of stimulation to be determined.

The amplitude of antennal responses to an odor is expressed as a percentage of the average amplitude of two responses to amyl acetate before and after the test stimulation. For each odor, the average amplitude response of 14–19 antennae was calculated. Data were analyzed by one-way or two-way ANOVA after arc sin $\sqrt{x}$ transformation.

Chemicals and Hair-Pencil Extracts Tested. Twelve *n*-alkanes, eight found in the hair-pencils and four analogs, from tetradecane (C_{14}) to pentacosane (C_{25}), were tested. Chemicals (15 mg) were placed on a 1- × 2-cm piece of Whatman filter paper and placed in a 6-cm-long × 0.7-cm-diameter glass cartridge that could be inserted into the airflow (the stimulator) when needed. Prior to use, cartridges were stoppered and kept at −18°C. One cartridge was used for 20 stimulations.

One minute before the stimulations, the cartridge containing the *n*-alkane to be tested was warmed for 30 sec to about 40–50°C until the compounds

liquefied. Melting the alkanes used in EAG was deemed necessary since, in a preliminary experiment on females with C_{16}, C_{17}, and C_{19} without warming, no antennal responses were observed.

An extract of hair-pencils in hexane also was studied by placing 60 hair-pencils (the equivalent of 30 males) on the filter paper of a stimulatory cartridge.

Pheromone Concentration. To evaluate the concentration of products sent towards the antenna during a stimulation, an adsorbant cartridge containing Tenax GC was fixed at the exit of the stimulator airflow to trap volatiles. Thirty stimulations per chemical were made with C_{16} and with C_{23}, and the trapped compound in each case was eluted from the adsorbant with 1 ml of diethyl ether, and 10 μl of eluant was injected into a gas chromatograph. The surface area of the peak obtained was compared to that obtained after chromatography under the same conditions (Auger and Ferary, 1994) of a known quantity of the *n*-alkane in ether solution.

Behavioral Observations. The effects of male pheromone on the behavior of conspecific males were observed in a 2-cm-diameter $\times$ 10-cm-long glass tube. This tube, split into two identical compartments by plastic mesh held in place by circular clips, could be placed at the opening of a 250-ml container holding ten 4- to 6-day-old virgin females. Airflow of 5 cm/sec was passed through the apparatus. A 4- to 6-day-old virgin male was placed in the upwind compartment of the tube and stimulated by calling females. The male pheromone source placed in the same upwind compartment was either another identical male, or a 2-cm^2 clean filter paper (control), or a 2-cm^2 filter paper containing 10 mg of C_{16} alkane. This test paper was introduced, either 18 hr before the experiment, or only 10 min before the experiment in order to avoid potential sensory adaptation towards C_{16}. Continued observation of the male(s) for 3 min enabled the mean wing fluttering duration per male to be measured. Repetitions where no male responded in the 3 min of observations were rejected.

In a second experiment, males were always placed in the downwind compartment. The effects of three alkanes, C_{17}, C_{18}, and C_{21} were tested separately as well as the $C_{17} + C_{21}$ mixture. Ten milligrams of each alkane, either pure or in hexane solution, was put on a filter paper (2 cm^2), and the mixture consisted of 5 mg of C_{17} and 5 mg of C_{21} alkanes. Each component or mixture was tested twice. The first time, the paper was placed in the upwind compartment without contact with the male, and the second time, in the downwind compartment with the male. The mean duration of fluttering per downwind male was calculated for 3 min in each of the eight experiments and was compared to the mean duration of fluttering of a control downwind male placed with a clean paper.

Another experiment carried out under the same conditions was intended to differentiate the action of male wing fluttering from the action of hair-pencil pheromone itself. Four groups of males were observed as follows: (1) a control

male isolated downwind without male pheromone; (2) a downwind male with an upwind undamaged male as pheromone source; (3) a downwind male with an upwind male whose wings were glued together with a drop of colorless nail varnish—the upwind male could move freely and extrude its hair-pencils but could not flutter its wings in response to female pheromone; and (4) a downwind male with an upwind male whose abdominal extremity was glued so that it could respond to female pheromone by moving freely and by wing fluttering, but could not extrude the hair-pencils.

Thirty repetitions of these three experiments were conducted. Data were analysed by nonparametric Kruskal-Wallis analysis of variance, or χ^2 test.

RESULTS

Concentrations of Products in Airstream to Antennae. The concentrations coming out of the stimulator airflow directed towards the antenna were estimated at 1.2×10^{12} molecules/cm^3 for C_{16} and 3×10^{11} molecules/cm^3 for C_{23}. These calculations were based upon the airflow (5 liters/min), the cartridge volume, the stimulation duration, and the results obtained from GC analyses. The concentration of C_{16} was four times higher than that of C_{23}.

Based upon the quantities of alkanes observed in the hair-pencils and the number of hair-pencils in the extract, the concentration of pheromonal compounds can be estimated at 2×10^9 molecules/cm^3 of air.

Stimulation Duration. To determine the optimal stimulation duration for EAG, consideration was given to two criteria—the mean and the coefficient of variation of the antennal response (Figure 1). The stimulation duration that seemed most interesting was 1.6 sec for the three groups of adults and the four *n*-alkanes tested. The response was often highest with a stimulation duration of 1.6 sec, and the coefficient of variation was frequently the lowest. Stimulation duration of 0.8 sec was eliminated because it triggered responses in which the means were lower than those obtained with a stimulation of 1.6 sec in eight cases out of 12, and the coefficients of variation were higher in eight of 12 cases.

Antennal Responses. All chemicals tested, as well as the hair-pencil extracts, elicited antennal responses in the three groups of adults studied (Figure 2). In males reared on leek, the EAG responses were significantly different ($P < 0.001$). The responses obtained with C_{16}, C_{19}, and C_{22} *n*-alkanes were higher than those obtained with *n*-alkanes that are not secreted by the insect (C_{14}, C_{15}, C_{24}, and C_{25}), and higher than with C_{17} or with the extract of male hair-pencils. Responses to other *n*-alkanes present in the hair-pencils (C_{18}, C_{20}, C_{21}, and C_{23}) only differed from responses to C_{15}, C_{24}, and C_{25}. In females reared on artificial diet containing added leek, responses were more homogenous but nevertheless

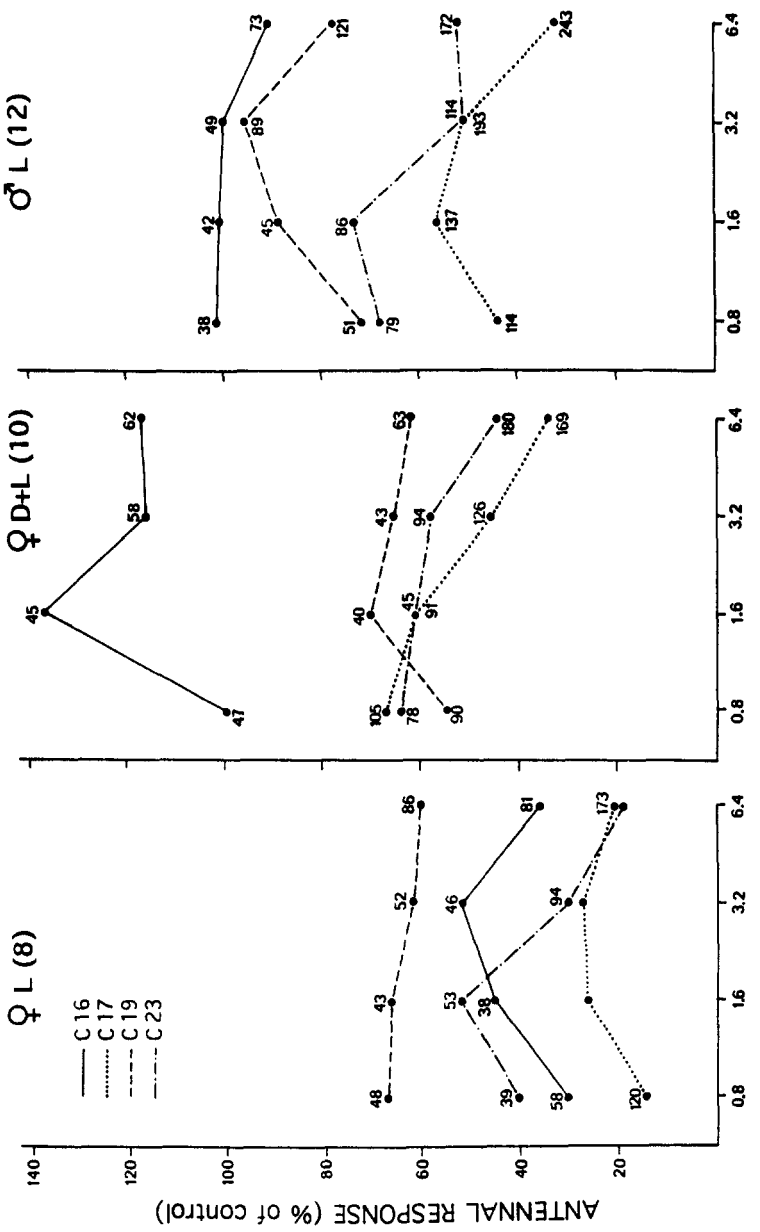

FIG. 1. Mean EAG responses of three groups of *A. assectella* adults for four alkanes of the male pheromone according to the duration of stimulation. The number of antennae tested is indicated in parentheses for each adult group. The value of the coefficient of variation is indicated for each mean. L: adults reared on leek; D+L: females reared on diet with leek.

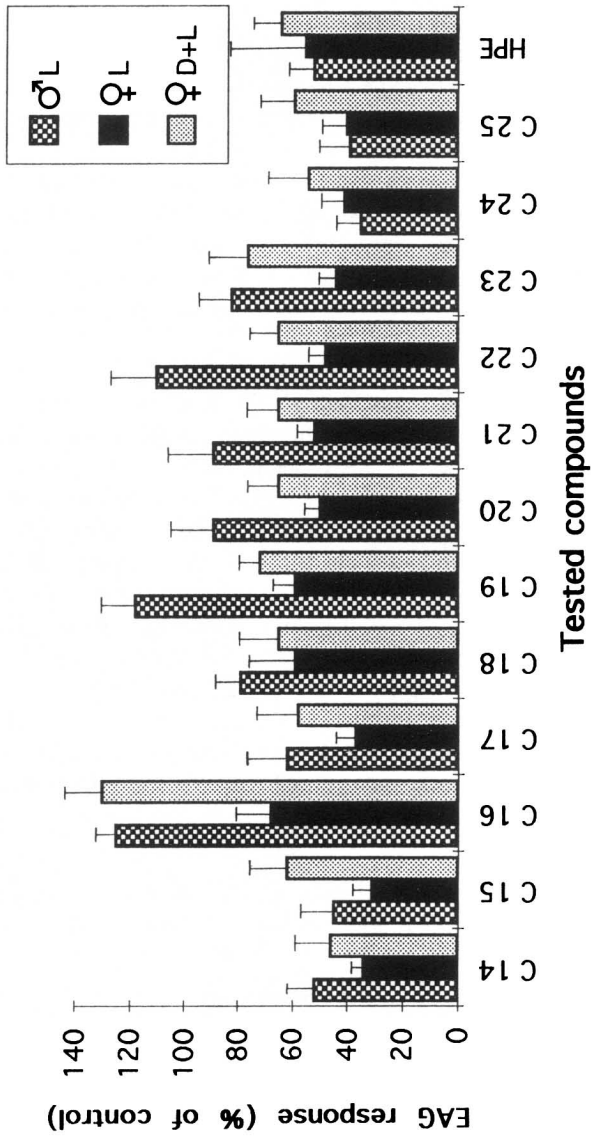

FIG. 2. Mean (±SEM) EAG responses of three groups of *A. assectella* adults for 12 alkanes and for an extract of male hair-pencils (HPE). L: adults reared on leek; D+L: females reared on artificial diet containing added leek.

different ($P = 0.004$) from females reared on leek. Responses obtained with C_{16} were significantly higher than those obtained with other *n*-alkanes but were similar to those obtained with C_{19} and C_{23}. Finally, in females reared on leek all of the responses were homogenous ($P = 0.078$). Only the response to C_{14} differed from those to C_{16} and C_{19}. Two-way analysis of variance indicated that females reared on artificial diet and males responded in a comparable way, while females reared on leek responded significantly less ($P < 0.001$).

As a whole, C_{16} was always the *n*-alkane triggering the strongest antennal responses, followed to a lesser degree by C_{19}, particularly in males. Responses to C_{16} were different from those caused by the *n*-alkanes that are absent from the male pheromone (C_{14}, C_{15}, C_{24}, and C_{25}). The EAG responses observed with the hair-pencil extracts, however, were not different from responses observed with the synthetic *n*-alkanes.

The average absolute values of EAG responses to C_{16} stimulation and to the first amyl acetate stimulation as well as the responses to the chemicals relative to amyl acetate were compared in the three groups of adults (Table 1). While the responses to C_{16} are statistically equal in the three types of adults, the responses to amyl acetate are stronger in females reared on leek than in males reared on leek, and females reared on artificial diet containing added leek.

Behavior of Males. When two males were placed together and subjected to female pheromone, it was rare for the two males to begin wing fluttering simultaneously. Almost always, one of them would react more quickly (hereafter designated as the first fluttering male) and would flutter its wings for a long time and in a violent manner while the second (designated as the second fluttering male) would hardly flutter its wings at all, sometimes making a few more or less isolated wing flaps. Often, after several homosexual mating attempts, second fluttering males would become immobile at the downwind end of the

TABLE 1. EAG RESPONSES OF THREE TYPES OF *A. assectella* ADULTS DURING FIRST STIMULATION WITH AMYL ACETATE (CONTROL) AND HEXADECANE (C_{16})[a]

	EAG response (mean ± SEM)		
	Males reared on leek	Females reared on leek	Females reared on diet
Amyl acetate	0.87 ± 0.07a	1.98 ± 0.26b	1.09 ± 0.11a
C_{16}	0.91 ± 0.08a	0.88 ± 0.11a	1.01 ± 0.15a

[a]The number of repetitions was 14–19. Means in the same row followed by the same letter do not differ at the $\alpha = 0.05$ threshold (Kruskal-Wallis ANOVA).

compartment. First fluttering males displayed the longest wing fluttering duration while second fluttering males had the shortest duration (Figure 3, left).

The five series of males (Figure 3, left) can be divided statistically into three groups based upon the presence or absence of male pheromone. The first group contains first fluttering males and the control males that both flutter for a long time in the presence of a clean paper. The second group contains the two series of males in the presence of a paper with C_{16} *n*-alkane with the treated paper put into position either 18 hr or 10 min before the experiment. Wing fluttering durations were not statistically different in these two series of males, but were different from the durations of the two series of the first group. Finally, there was a third group with the second fluttering males, different from all of the above male series, that fluttered little and stopped moving at the 3rd min. The difference in the number of repetitions carried out to obtain 30 males with wing fluttering in each series is variable, but never significant. In control males with clean paper, 16 of 46 males tested did not respond to female pheromone,

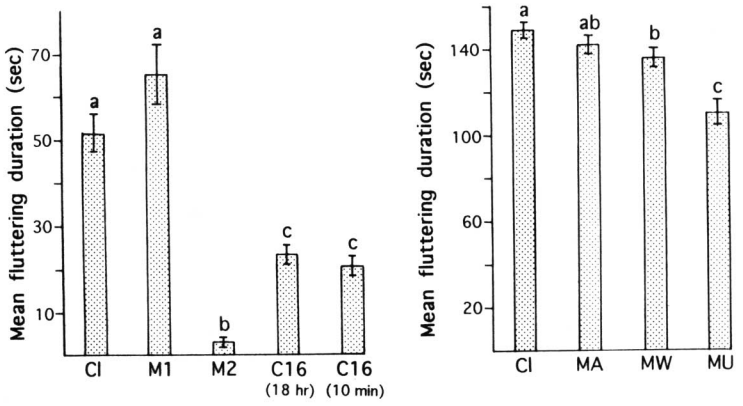

FIG. 3. Mean wing fluttering duration (in sec, ±SEM) of various groups of *A. assectella* males placed in the presence of a male pheromone source. Left: Upwind males. M1: First male to flutter when two males are together; M2: second male to flutter when two males are together; Cl: control males placed with a clean paper; C16 (18 hr): males exposed to a paper containing C_{16} alkane for 18 hr before the beginning of observation; C16 (10 min): males exposed to a paper containing C_{16} alkane for 10 min before the beginning of observation. Right: Downwind male in the presence of an other male upwind. Cl: control downwind male without male pheromone; MA: the upwind source of stimulus is a male with glued abdomen; MW: the upwind source of stimulus is a male with glued wings; MU: the upwind source of stimulus is an undamaged male. Means followed by the same letter do not differ at the $\alpha = 0.05$ threshold (Kruskal-Wallis ANOVA).

while in males exposed to with C_{16} (10 min before the experiment) 30 of a total of 60 did not respond ($P = 0.117$).

Behavior of Downwind Males in the Presence of Alkanes. Mean wing fluttering duration (Table 2) was significantly higher in control males than in males in the presence of *n*-alkanes. Furthermore, the decrease in fluttering duration was highest when the males were in the presence of C_{17} either alone or in a mixture than in the presence of C_{18} or C_{21}. The more volatile alkanes seemed to be the most active, but their perception by olfaction without contact was less effective than perception by olfaction plus contact. This difference was less clear and disappeared with the larger, less volatile *n*-alkanes tested. Thus, if there is a possibility of contact with C_{17} or C_{18}, males have a significantly shorter wing fluttering duration than those males with no possibility of this contact (Table 2).

The presence of *n*-alkanes totally inhibits the sexual behavior of certain males. While all the control males fluttered in response to female pheromone, several individuals in the other groups were incapable of responding to female pheromone in the presence of an *n*-alkane. This was particularly true for males able to contact C_{17} (Table 2).

Behavior of Downwind Males in the Presence of Males with Glued Wings or Abdomen. The presence of an undamaged male upwind brought about a

TABLE 2. WING FLUTTERING DURATION BY DOWNWIND *A. assectella* MALES AND PERCENTAGE OF MALES WITH NO RESPONSE TO FEMALE PHEROMONE[a]

	Wing fluttering duration (sec, mean ± SEM)		Males: Total tested (*N*)	Males: No response (%)
Control	148.4a	2.9	30	0a
C_{17} (D)	75.5b	7.7	55	45.4b
C_{17} (U)	100.4d	7.8	38	21.1c
C_{18} (D)	100.1d	7.5	45	33.3bc
C_{18} (U)	120.0c	3.8	36	16.7c
C_{21} (D)	116.4c	5.8	39	23.1c
C_{21} (U)	119.2c	6.2	41	26.8bc
$C_{17} + C_{21}$ (D)	88.2b	6.5	43	30.2bc
$C_{17} + C_{21}$ (U)	86.7b	6.7	37	18.9c

[a] C_{17} = heptadecane; C_{18} = octadecane; C_{21} = henicosane. Data in the same column followed by the same letter do not differ at the $\alpha = 0.05$ threshold (χ^2 test and Kruskal-Wallis ANOVA). Downwind males were in the presence of a paper with pure alkane or with a mixture of two alkanes placed in the downwind (D) or upwind (U) compartment.

significant lessening of the wing fluttering duration of the downwind male. The presence upwind of a male with either the wings or the abdomen glued led to a wing fluttering duration of the downwind male that was midway between the former and that of control males not subjected to male pheromones (Figure 3, right). However, when the abdominal extremity was glued, the wing fluttering duration was not significantly different from that of the controls, but there was a significant difference between control males and downwind males in the presence of a pheromone source from upwind males with wings glued.

DISCUSSION

The different alkanes constituting the male pheromone of *A. assectella* are clearly perceived by the male and female antenna of this species at concentrations of around 10^{11} and 10^{12} molecules/cm^3 of air. These concentrations are comparable to those normally used in electrophysiology and in olfactometry in several species (Kaissling, 1971, 1987; Ma and Visser, 1978), which generally extend from 10^{12} to 10^{16} molecules/cm^3 for plant chemicals, frequently with higher responses above 10^{13}. This latter concentration has been used for a study of the effects of plant odors on the behavior of *A. assectella* (Lecomte and Thibout, 1981). The hair-pencil chemicals are perceived at a concentration of about 10^9 molecules/cm^3 of air, a concentration often used in studies of pheromone perception.

The antennal responses appear globally higher for the *n*-alkanes present in the hair-pencils (C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, C_{21}, C_{22}, C_{23}) than for C_{14}, C_{15}, C_{24}, and C_{25}, which are absent from the hair-pencils. The intensity of the EAG response is not dependent solely upon volatility of the *n*-alkanes, although volatility appears to be important. The *n*-alkanes C_{14} and C_{15}, which are absent from the pheromone, give antennal responses that are not very strong, in contrast to C_{16}, which gives the most intense responses and is present in very low quantity in the pheromone (Thibout and Auger, 1995). C_{16} also has a strong behavioral effect on females. Moreover, C_{17} is certainly the *n*-alkane that gives the lowest EAG amplitude despite being found in high quantities and having a strong behavioral effect on females (Thibout et al., 1994) as well as males. In the male pheromonal compounds of *A. assectella*, there is no correlation between quantity present, olfactory perception, and behavioral activity in females as in males. The males respond more strongly than females. In a great majority of species studied, the antennal responses of males to the male-produced wing or abdominal pheromones are comparable to (Schneider and Seibt, 1969; Grant, 1971; Palaniswamy et al., 1979; Burger et al., 1993) or weaker than responses of females (Jacquin et al., 1991; Pivnick et al., 1992). *Heliothis virescens* and *Pseudaletia unipuncta* are particularly interesting since these two species have been the

object of several EAG studies with differing results. In *H. virescens*, one study has shown that the two sexes respond similarly to abdominal scent-brush volatiles (Grant, 1971), while a second study concluded that those volatiles, particularly (*Z*)-9-tetradecenal, induced stronger antennal responses in males than in females (Jacobson et al., 1984). *P. unipuncta* males and females make similar EAG responses to benzaldehyde and to benzyl alcohol identified in the abdominal scent brushes (Grant et al., 1972). Some authors, however, found that males responded better to benzaldehyde than females (Seabrook et al., 1979; Fitzpatrick et al., 1989). In these two species, as in *A. assectella*, male pheromones inhibited the sexual behavior of conspecific males (Hirai et al., 1978; Hendricks, 1976), and also elicited stronger antennal responses in males than in females.

A comparison between *A. assectella* females reared on leek for less than a year with those reared on artificial diet for a dozen years shows that the latter respond in EAG to male pheromone in a comparable way to males, and significantly more than females reared on the host plant. There is no loss of sensitivity in females to these odors due to high-density rearing conditions on an artificial diet in the laboratory. In these conditions of high density, *A. assectella* males, during mating, continue to use their hair-pencils, which might retain a high selective value due to strong competition. In other species, inbreeding conditions in the laboratory caused a loss of function in the hair-pencils (Birch et al., 1990; Lofstedt et al., 1989).

With the exception of the work of Burger et al., (1993) where no information is given on the absolute value of antennal responses, in all the EAG studies concerning the perception of male pheromones in Lepidoptera (Schneider and Seibt, 1969; Grant, 1971; Grant et al., 1972; Palaniswamy et al., 1979; Seabrook et al., 1979; Jacobson et al., 1984; Fitzpatrick et al., 1989; Jacquin et al., 1991), responses are expressed in millivolts and not relative to a standard. In absolute value, in *A. assectella* as in the majority of species studied, the responses of males and females to male pheromone are similar. The best responses relative to males reared on leek or females reared on artificial diet could not therefore be the consequence of an absolute strongest response to male pheromone as previously discussed, but a weaker response to the compound taken as control. This second hypothesis is probable as regards females reared on artificial diet without real contact with the host plant for generations, which could have lost their sensitivity to certain natural odors. The consequence of inbreeding would be preferentially felt in females.

In the experiment with only one compartment, the first male to flutter did so at the beginning of the experiment and continued to do so during almost all the observation time, while the second male to flutter, which began later, only fluttered for a few seconds during the observation time. This phenomenon indicates an inhibition of the sexual behavior of the second male. This inhibition seems to be at least partially caused by male pheromone since the fluttering

duration of a male placed in the presence of C_{16} alkane is significantly less to that of first male or even of the control male in the presence of clean paper. The inhibition of the male in the presence of C_{16} is, however, weaker than that exerted by the first male on second male. This significant difference can come from variations in pheromone concentration, the absence of certain pheromonal compounds, since only C_{16} is used, or the absence of certain physical or other stimuli linked to wing fluttering.

In the two experiments with both compartments, the inhibitory role of male pheromone and their alkanes on the sexual behavior of conspecific males is clearly confirmed. Comparisons between the three behavioral experiments show that the inhibition is greater when two males are together than when the two males are separated. Similar results are obtained with alkanes used in the pure state or mixed. Perception by olfaction of male pheromone and its constituents is thus sufficient to cause sexual inhibition of the receptive male, but possible contact with the pheromone source leads to a stronger inhibition. It seems that the most volatile compounds, C_{16}, C_{17}, and C_{18} are the most active. There is a significant difference in activity for C_{17} and C_{18} between perception by olfaction alone and by olfaction plus contact. Synergism does not seem to exist between the various alkanes since the results obtained with the mixture $C_{17} + C_{21}$ are intermediate between those of the two products taken in isolation.

The presence upwind of males with their wings or their abdominal extremities glued clearly differentiates the effect of wing fluttering from that of the hair-pencil pheromone. When the wings are glued, they cannot flutter but the male can extrude its hair pencils. In this case, a significant inhibition of the downwind male appears in comparison to the control male. In contrast, when the hair-pencils cannot be extruded but wing fluttering is possible, there is no significant difference between the downwind males and the control males. Wing fluttering has, however, a slight but indirect effect. It improves the effect of the male pheromone since a healthy male combining the two stimuli has a stronger inhibitory effect on the downwind male than if it were only able to flutter its wings.

The role of male pheromones as inhibitors of sexual behavior of conspecific males has been put forward previously but has yet to be generalized in the species studied. The presence of wing androconial scales of *Zizeeria maha argia* males lessens the mating attempts of other males of the same species (Wago, 1978). In *H. virescens*, after having thought that the pheromone of male abdominal scent brushes could inhibit the production of female pheromone (Hendricks and Shaver, 1975), Hendricks (1976) concluded that there was a disruption of male sexual behavior. This effect could be due to (Z)-9-tetradecenal produced in large quantities by the brushes of *H. virescens* males (Jacobson et al., 1984). Inhibition of male sexual behavior by its own pheromone also has been described in several species of *Yponomeuta* (Hendrikse et al., 1984), with inhibition rein-

forced by the wing fluttering of the emitting male. In *Adoxophyes orana*, the odor of wing fluttering males inhibits wing fluttering of other males placed downwind in an air current (Bijpost et al., 1985). Wing fluttering alone without male odor has no effect on other males. Palmitic acid produced by the males may be one of the main compounds responsible for inhibition (Den Otter et al., 1989). An inhibitory role for male pheromones in Lepidoptera has not been observed in all species studied. In *Mamestra brassicae* the inhibition has not been clearly shown (Toth, 1982), but the number of repetitions was low, (less than 14). Finally, in *Pseudaletia unipuncta*, two studies reached diverging conclusions. Sexual behavior of males of this species, which have abdominal scent brushes, brings about a lessening of sexual response in other males placed downwind in an air current (Hirai et al., 1978). However, in a flight tunnel, males of *P. unipuncta* are neither repelled nor inhibited by the presence of male scent brushes when presented with a source of female pheromone (Fitzpatrick et al., 1988). While the results indicate that males are not repelled by pheromone of their own species in *P. unipuncta*, the conclusions concerning inhibition of sexual behavior, even though the male moves towards the source of female pheromone, seem less certain. When the male comes into contact with female pheromone placed upwind of male pheromone, it is no longer subjected permanently to male pheromone.

The inhibitory effect of male pheromone on conspecific males over a short distance appears to be a widespread phenomenon in species of Lepidoptera that do not form a lek before mating. Other factors linked to male sexual activity, such as vibrations, contact, and movements, must also have an effect as seems to be the case in the *Hyponomeuta* (Hendrikse et al., 1984) and in *A. assectella*. It is possible that wing fluttering, as well as having a probable role in establishing an air current, has a role in the volatilization of the alkanes of the hair-pencils by raising the temperature. Wing-fluttering raises the internal temperature of an insect (Heinrich, 1971), and a slight heating of the alkanes of *A. assectella* favors the perception of these compounds by the antennae of males and females.

Many insects produce alkanes. In Formicidae, for example, the size of the alkanes seems to vary depending on their glandular origin. The number of carbon atoms in cuticular alkanes of ants frequently is between 23 and 33 (Bonavita-Cougourdan et al., 1987; Nowbahari et al., 1990; Hefetz et al., 1992), as in the cuticular alkanes of other insects (De Renobales and Blomquist, 1983). In the Dufour gland of ants, however, the number of carbon atoms in the alkanes is generally between 12 and 23 (Keegans et al., 1992; Brand and Mpuru, 1993). The alkanes in the male pheromone of *A. assectella* have 16–23 carbons and are comparable to those identified as trail pheromones in some ants (Jackson and Morgan, 1993). Their information role could, like the trail pheromones, be maintained over a prolonged time. During *A. assectella* sexual behavior, there is contact between the abdominal extremity of the male (where the hair-pencils

are found) and the female's body (Thibout, 1972b). Thus, compounds from the male could be deposited on the female, compounds whose inhibitory effect on conspecific males has been demonstrated in this study. In this way the compounds could favor monogamy, which has been observed in this species (Thibout, 1975).

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PUTATIVE CHEMICAL SIGNALS FROM WHITE-TAILED DEER (*Odocoileus virginianus*): SOCIAL AND SEASONAL EFFECTS ON URINARY VOLATILE EXCRETION IN MALES

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Abstract—Urine samples collected from dominant and subordinate male white-tailed deer during the breeding and nonbreeding season were analyzed by combined gas chromatography–mass spectrometry (GC-MS). Fifty-five volatiles were found in measurable quantities. Ketones were most numerous, followed by alcohols and alkanes. Nine compounds were common to both dominants and subordinates during the breeding season. Of these nine, three were present in higher concentrations in dominants, and six were higher in subordinates. During the breeding season, nine compounds were found exclusively in the urine of dominants, whereas 19 compounds were found exclusively in the urine of subordinates. Concentrations of several compounds were dependent on the time of year (breeding vs. nonbreeding season). Differences in compound presence and concentration may produce a rank-specific odor, although we suggest that differing concentrations of these suites of compounds may be more important for the identification of social status than the presence of individual compounds. Since mature male white-tailed deer urinate on their tarsal glands frequently during the breeding season, this behavior may allow a deer to simultaneously scent-mark its environment and carry intraspecific cues indicative of social status.

Key Words—Chemosignals, *Odocoileus virginianus*, scent communication, semiochemical, urine, volatiles, white-tailed deer.

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INTRODUCTION

The scent communication system of white-tailed deer (*Odocoileus virginianus*), which is poorly understood at present, has been the subject of several studies in recent years. Compounds produced by various integumentary structures, such as the interdigital, forehead, and tarsal glands, are either carried on the body or deposited into the environment as olfactory signposts (Miller et al., 1987a, b; Marchinton et al., 1990; Gassett et al., 1996, 1997). Urine-washing is a common method of scent application as well as a threat display in many mammals (Johnson, 1983; Albone, 1984). Behavioral observations of male white-tailed deer indicate that urine plays a vital role in olfactory communication (Moore and Marchinton, 1974; Miller et al., 1987b; Marchinton et al., 1990). Rub-urination, a behavior whereby a deer urinates on its tarsal glands while rubbing them together, can be a marking or agonistic behavior, depending on context. During the breeding season, mature males produce scrapes (a scent mark) by marking a low, overhanging limb with their head, pawing a shallow depression in the leaf litter/soil beneath the limb, and then rub-urinating over the tarsal glands into the depression (Moore and Marchinton, 1974). This mark may serve as a communication to potential rivals (Coblentz, 1976) or as a priming pheromone to induce the termination of seasonal anestrus of females (Miller et al., 1991). Mature males also will perform rub-urination in the presence of another male as a display of dominance. This behavior may provide visual as well as olfactory cues to other males.

Urine is a concentrated source of volatiles that are deposited into the environment upon excretion. Changes in steroid production resulting from heirarchy establishment in preparation for the breeding season may affect the production of urinary volatiles. Differences in urinary volatiles deposited into the environment or retained on the tarsal gland may identify an individual as dominant or subordinate. In this study, we identified profiles of volatile compounds from urine samples of dominant and subordinate bucks during the breeding and non-breeding season.

METHODS AND MATERIALS

Subject and Sample Collection. Urine samples were collected from three adult (4–5 years old) dominant and three adult (3–4 years old) subordinate male deer. Deer were housed at the University of Georgia's Whitehall Deer Research Facility. A commercial feed (Omolene 300, Purine Mills, Inc.), and bermuda grass (*Cynodon dactylon*) or alfalfa (*Medicago sativa*) hay was available. Deer were members of various social groups maintained in 1-ha pens. Does and fawns were present to provide stimuli to induce the establishment of a dominance hierarchy. Dominance was determined by following the methods of Lehner (1979).

Urine samples were collected from all males during the breeding season (October–January) and nonbreeding season (April–July). Study animals were anesthetized using a mixture of xylazine hydrochloride and ketamine hydrochloride as described by Mech et al. (1985). The penis of each male then was placed in a sterile collection tube. Males remained sedated until urination occurred (≤ 15 min). Anesthesia was reversed with yohimbine hydrochloride. Immediately after collection, urine was divided into three 1.5-ml aliquots and frozen at $-20°C$ until analyzed.

Procedure. Urinary volatiles were concentrated onto a porous polymer (Tenax GC) with a modified headspace technique developed by Novotny et al. (1974) and McConnell et al. (1979). To give each sample (1.5 ml) the same high ionic strength, 0.4 g of $(NH_4)_2SO_4$ was added. Next, the sample was absorbed on 0.6 g of glass wool. Volatiles then were purged from the glass wool by passing purified helium gas through it at a flow rate of 30 ml/min for 30 min at 50°C. The volatiles then were passed through a cooled condenser, installed to reduce water mist originating from the sample, and trapped onto a 4-mg Tenax GC precolumn packed into the glass injection port liner of a gas chromatograph.

After the headspace sampling procedure, the precolumn was inserted into the modified injection port of a Hewlett-Packard model 5980A gas chromatograph–mass spectrometer (GC-MS). The volatiles were desorbed at 200°C and retrapped cryogenically for 15 min to compress the desorbed sample at the column inlet. The column used was a glass capillary column (30 m × 0.25 mm ID) that had been statically coated with UCON-5-HB-200 (Supelco Inc., Bellefonte, Pennsylvania). Column temperature was programmed from 30 to 160°C at a rate of 2°C/min. Volatile constituents were identified through their mass spectra, using electron impact ionization at 70 eV. Peak areas were evaluated with a GC-MS data station. The identification of volatile components was verified by retention time measurements of authentic compounds.

Statistical Analysis. Statistical comparison of the concentration of excreted volatiles (expressed by integrated peak areas) was performed by using t test ($P < 0.02$) and one-way analysis of variance (F at $P < 0.05$) with the Tukey test for equal sample size ($P < 0.02$). Because of the heteroscedastic data, the inequality of variance was corrected by logarithmic transformation of original X values (integrated peak areas in arbitrary units) to $X' = \log (X + 1)$ values (Zar, 1984).

RESULTS AND DISCUSSION

Fifty-five volatiles occurred in male urine in measurable quantities. The volatiles identified in the urine of male deer belongs to the alcohol, aldehyde, furan, ketone, nitrile, alkene, alkane, thiol ester, disulfide, benzene, ether, ketal,

and amine classes of compounds. These classes of compounds have been reported from the urine of other mammals, including mice, voles, female deer, and humans (Albone, 1984; Novotny et al., 1990; Jemiolo et al., 1995). Figure 1 is a typical gas chromatogram that represents the volatile profile of voided dominant urine taken during the breeding season. Not all peaks representing the identified compounds are numbered.

Urine of dominant males taken during the breeding season contained nine volatile compounds not found in the urine of subordinate males (either season) or in the urine of dominants during the nonbreeding season (Table 1). However, urine of subordinates contained more volatiles than urine of dominant males (Table 2). Nineteen compounds were present only in the urine of subordinates during the breeding season. Concentrations of these compounds were relatively high, at a range of 1.20–3.23 log of the peak areas. The urine of dominant and subordinate males from the breeding season contained nine common compounds (Table 3). Interestingly, six compounds were present at higher levels ($P < 0.02$) in the urine of subordinates, whereas only three were found in higher ($P < 0.02$) concentrations in dominants. Several volatiles were common to all samples regardless of dominance or breeding season. However, concentrations of these compounds depended on social or seasonal factors (Table 4). In general, male urine from the nonbreeding season contained volatiles in lower concentrations than urine from the breeding season. Eight ketones (3-buten-2-one; 2-pentanone; 4-methyl-2-pentanone; 2,3-pentanedione; 2-hexanone; mesityl oxide; 4-heptanone; and 2-heptanone) increased in concentration ($P < 0.02$) from the nonbreeding to the breeding season, regardless of social status. Only one compound (decanal) was found at higher levels during the nonbreeding season. During the nonbreeding season, concentrations of most compounds (72%) did not depend on the male's social status from the previous breeding season. Five compounds [3-methylbutanal, α-terpineol, a terpene ether, an unsaturated cyclic ether, and 2-(5-methyl-5-vinyl-tetrahydrofuran-2-yl) propanol] were found in higher levels ($P < 0.02$) in former dominants than in former subordinates. The concentration of these compounds remained high throughout the current breeding season. During the breeding season, the urine of subordinate males contained two compounds (3-pentanone and 3-hexanone) at higher concentrations than those in dominant male urine. These ketones were present in dominant male urine but exhibited a significant ($P < 0.02$) drop in concentration during the breeding season.

Our studies of urinary volatiles from male (the present study) and female white-tailed deer (Jemiolo et al., 1995) indicate that the presence and concentration of urinary compounds depend on season, reproductive status, and social rank. We characterized 63 compounds in the urine of females (Jemiolo et al., 1995) and 55 in the urine of males. Twenty-seven of these compounds were

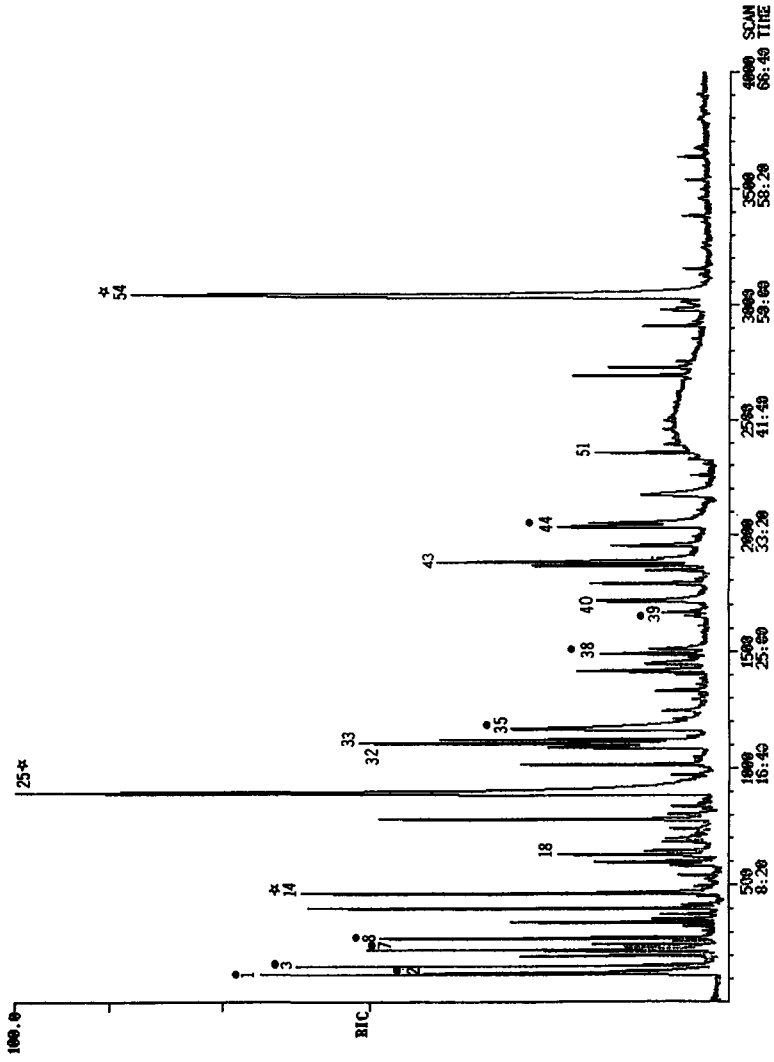

FIG. 1. GC-MS profile of volatile compounds from voided urine of a dominant male white-tailed deer. Numbered peaks marked with filled dots represent compounds characteristic of dominant urine only. Numbered peaks marked by star were present in the dominant male urine in higher concentrations than in the urine of subordinates. Numbered peaks not marked were present in the dominant urine in lower concentrations than in the subordinate urine.

TABLE 1. PEAK AREAS OF VOLATILE COMPOUNDS FOUND EXCLUSIVELY IN URINE OF DOMINANT MALE WHITE-TAILED DEER (N = 3) DURING BREEDING SEASON

Compound class	Peak number	Structure	Integrated peak areas (mean ± SEM)[a]
Alkene	1	1,3-pentadiene	1.83 ± 0.02
Alkanes	2	hexane	1.62 ± 0.03
	3	2-methylhexane	1.23 ± 0.01
Nitrile	7	isobutyronitrile	1.04 ± 0.02
Furan	8	2,5-dimethylfuran	1.31 ± 0.02
Alcohols	35	3-methyl-2-buten-2-ol	2.08 ± 0.02
	38	1-hexanol	1.25 ± 0.02
	44	3-methyl-1,3-butanediol	1.89 ± 0.04
Ketone	39	MW 138	0.64 ± 0.00

[a]The value of integrated peak areas X (in arbitrary units) was transformed to $X' = \log (X + 1)$.

TABLE 2. PEAK AREAS OF VOLATILE COMPOUNDS EXCLUSIVELY IN URINE OF SUBORDINATE MALE WHITE-TAILED DEER (N = 3) DURING THE BREEDING SEASON

Compound class	Peak number	Structure	Integrated peak areas (mean ± SEM)[a]
Alcohol	23	1-penten-3-ol	1.60 ± 0.02
Alkanes	6	octane	1.45 ± 0.01
	20	decane	2.12 ± 0.01
Alkenes	27	α-phellandrene	2.15 ± 0.08
	30	limonene	1.92 ± 0.08
	31	β-phellandrene	2.80 ± 0.03
Thiol Ester	28	ethyl thiolacetate	1.49 ± 0.04
Ketones	26	6-methyl-2-heptanone	2.00 ± 0.02
	36	3-ethylcyclopentanone	2.49 ± 0.03
	49	3-pentylcyclopentanone	1.92 ± 0.05
	46	6-methyl-3,5-heptadien-2-one	3.23 ± 0.04
	50	6-methyl-2-cyclohexene-1-one	2.44 ± 0.02
	52	2,3-dihydro-carvone	2.39 ± 0.05
	55	a dimethylacetophenone	2.93 ± 0.06
Furan	48	2-butyrylfuran	2.33 ± 0.02
Disulfide	13	dimethyl disulfide	1.20 ± 0.02
Amine	24	2,6-dimethylpyridine	1.53 ± 0.05
Benzene	29	iso-butylbenzene	2.43 ± 0.06
Ketal	47	MW 154	3.14 ± 0.07

[a]Value of integrated peak areas X (in arbitrary units) was transformed to $X' = \log (X + 1)$.

TABLE 3. PEAK AREAS OF VOLATILE COMPOUNDS COMMON TO URINE OF DOMINANT (N = 3) AND SUBORDINATE (N = 3) MALE WHITE-TAILED DEER DURING BREEDING SEASON

Compound class	Peak number	Structure	Integrated peak areas (mean ± SEM)[a]	
			Dominant	Subordinate
Alcohols	25	3-methyl-3-buten-1-ol	3.94 ± 0.01[b]	2.55 ± 0.00
	33	2-methylenepentanol	2.30 ± 0.03	3.08 ± 0.03[b]
	43	4-methyl-1,4-butanediol	1.55 ± 0.04	2.64 ± 0.03[b]
Alkane	18	2,2,4,6,6-pentamethylheptane	1.34 ± 0.02	2.05 ± 0.15[b]
Aldehyde	40	benzaldehyde	1.50 ± 0.02	2.00 ± 0.01[b]
Nitrile	54	phenylacetonitrile	2.95 ± 0.02[b]	2.40 ± 0.00
Amine	14	3,4,5,6-tetrahydropyridine	2.58 ± 0.00[b]	1.32 ± 0.08
Ketone	51	dihydro-carvone	1.23 ± 0.00	3.25 ± 0.04[b]
Unknown	32	unidentified	1.69 ± 0.01	2.68 ± 0.02[b]

[a]The value of integrated peak areas X (in arbitrary units) was transformed to $X' = \log (X + 1)$.
[b]The marked means in the same row are significantly higher from unmarked means; $t_{0.02}(2)4 = 3.74$.

common to both sexes. Thirty-six occurred in females only, and 28 were present exclusively in males. Alcohols, aldehydes, alkanes, alkenes, amines, ethers, furans, and ketones occurred in the urine of either sex. However, thiol esters, benzene, ketals, disulfides, and nitriles were found in male but not in female urine. Phenols were found in female but not male urine (Table 5).

White-tailed deer rely on odor production and their sense of olfaction to facilitate intraspecific communication. Semiochemical cues likely signal hierarchal status, reproductive condition, and individual recognition. Because urine is a primary medium for excreting metabolized hormones, it may play a role in the determination and/or establishment of social rank. Additionally, the high degree of variability in urinary volatiles also may aid in individual recognition. Just prior to the onset of the breeding season, dominant male deer typically exhibit an increase in the concentration of circulatory androgens, which are eventually metabolized and excreted in the urine. Many of the by-products of the action of these steroids, as well as the excreted steroid metabolites, are reflected in the urinary volatiles. We suspect that these high concentrations of compounds common to both dominants and subordinates, and the nine compounds exclusive to dominants, are the result of increased levels of circulating androgens.

TABLE 4. URINARY VOLATILE DEPENDENCY ON REPRODUCTIVE SEASON, SOCIAL STATUS, AND ENDOCRINE STAGE OF MALE WHITE-TAILED DEER

Compound class	Peak number	Structure	Volatile compound level[a]			
			Nonbreeding		Breeding season	
			Subordinate	Dominant	Subordinate	Dominant
Aldehydes	5	3-methylbutanan	1	2	2	3
	45	decanal	2	2	1	1
Alcohols	19	1-butanol	1	1	1	1
	41	2-(5-methyl-5-vinyl tetrahydrofuran-2-yl)propanol	1	2	2	3
	53	alpha-terpineol	1	2	2	3
Ketones	4	3-buten-2-one	1	1	2	2
	9	2-pentanone	1	1	2	2
	10	3-pentanone	2	2	3	1
	11	4-methyl-2-pentanone	1	1	2	2
	12	2,3-pentanedione	1	1	2	2
	15	3-hexanone	2	2	3	1
	16	2-hexanone	1	1	2	2
	17	mesityl oxide	1	1	2	2
	21	4-heptanone	1	1	2	2
	22	2-heptanone	1	1	2	2
	37	6-methyl-5-hepten-2-one	1	1	1	1
Ethers	34	a terpene ether	1	2	2	3
	42	unsaturated cyclic ether	1	2	2	3

[a] $1 < 2 < 3$: significantly higher at the level $P < 0.02$; $F(3,8)$.

TABLE 5. DISTRIBUTION OF COMPOUND CLASSES PRESENT IN ALL TYPES OF URINE FROM MALE AND FEMALE WHITE-TAILED DEER

Compound class	Distribution (%) of class of compounds	
	Female	Male
Thiol ester	0	2
Alcohol	14	18
Aldehyde	21	5
Alkane	8	9
Alkene	18	7
Amine	1	4
Benzene	0	2
Ketal	0	2
Disulfide	0	2
Ether	1	4
Furan	3	4
Ketone	30	36
Nitrile	0	4
Phenol	1	0
Unidentified	3	2

The mechanism behind the higher concentrations and larger numbers of exclusive compounds in subordinates during the breeding season is unclear. However, it is likely that the overall hormone profile controls the production and relative concentration of urinary volatiles. The excess of exclusive volatiles and the increased concentrations in subordinates may result from lower androgen levels and increased glucocorticoid levels, and result in a different odor from that produced by dominants.

Few other studies have investigated urinary volatile profiles in cervids. In a study of volatile substances from red deer (*Cervus elaphus elaphus*), Bakke and Figenschou (1990) did not observe any systematic variation in volatiles among seasons. However, their analytical methodology differed from ours. Previously, we reported that urinary volatile profiles of female white-tailed deer are hormone-dependant and change according to the stage of the reproductive cycle (Jemiolo et al., 1995). The current study suggests that volatile profiles from male white tails likewise may be dependent on hormonal as well as social status.

During the breeding season, volatile compounds occurring in the urine may help establish dominance, attract mates, delineate territory, or identify individuals. Additionally, males urinate onto their tarsal glands. This rub-urination

provides a mechanism by which a buck can simultaneously mark his body and surroundings with a similar scent. In black-tailed deer (*O. hemionus columbianus*), Muller-Schwarze et al. (1978) presented evidence that a urinary lactone, (Z)-6-dodecen-4-olide, is selectively retained on the tarsal gland and is involved in social communication. This compound has not been reported from male white-tailed deer (Albone, 1984), and was not observed in our study.

This study documents for the first time that urinary volatiles in male whitetails are dependent on season, social status, and potentially on hormonal status. Additional behavioral research is needed to determine which compounds are important in communicating socially significant information. The study also provides chemical background for additional chemical analyses and behavioral bioassays for screening of biologically important compounds. Since the tarsal gland plays host to a wide variety of microorganisms (Gassett, unpublished), the role of bacteria in the conversion of urinary metabolites and in production of additional volatiles of potential biological importance should be investigated.

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EFFECTS OF MICROBIAL UTILIZATION OF PHENOLIC ACIDS AND THEIR PHENOLIC ACID BREAKDOWN PRODUCTS ON ALLELOPATHIC INTERACTIONS

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Abstract—Reversible sorption of phenolic acids by soils may provide some protection to phenolic acids from microbial degradation. In the absence of microbes, reversible sorption 35 days after addition of 0.5–3 μmol/g of ferulic acid or *p*-coumaric acid was 8–14% in Cecil A_p horizon and 31–38% in Cecil B_t horizon soil materials. The reversibly sorbed/solution ratios (r/s) for ferulic acid or *p*-coumaric acid ranged from 0.12 to 0.25 in A_p and 0.65 to 0.85 in B_t horizon soil materials. When microbes were introduced, the r/s ratio for both the A_p and B_t horizon soil materials increased over time up to 5 and 2, respectively, thereby indicating a more rapid utilization of solution phenolic acids over reversibly sorbed phenolic acids. The increase in r/s ratio and the overall microbial utilization of ferulic acid and/or *p*-coumaric acid were much more rapid in A_p than in B_t horizon soil materials. Reversible sorption, however, provided protection of phenolic acids from microbial utilization for only very short periods of time. Differential soil fixation, microbial production of benzoic acids (e.g., vanillic acid and *p*-hydroxybenzoic acid) from cinnamic acids (e.g., ferulic acid and *p*-coumaric acid, respectively), and the subsequent differential utilization of cinnamic and benzoic acids by soil microbes indicated that these processes can substantially influence the magnitude and duration of the phytoxicity of individual phenolic acids.

Key Words—Allelopathy, phenolic acids, sorption, microbial utilization, soil extractions, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid.

INTRODUCTION

Microbial metabolism is an important factor in determining the magnitude and duration of allelopathic interactions involving phenolic acids present in soils

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through the release of phenolic acids from organic residues or through the reduction of soil concentrations of phytotoxic phenolic acids (Rice, 1984; Dao, 1987; Elliott and Cheng, 1987; Siqueira et al., 1991; Cheng, 1995; Blum, 1995, 1996). The research described here focuses on microbial metabolism of available (i.e., free and reversibly sorbed) phenolic acids in soil, with emphasis on the accessibility of reversibly sorbed phenolic acids to microbes and the ways in which the initial phenolic acid breakdown products generated by microbes may influence the magnitude and duration of the phytotoxicity of phenolic acids in soils.

Dalton (1989) suggested that reversible sorption of phenolic acids may provide some protection to phenolic acids from microbial degradation. Since free phenolic acids generally have a short half-life in most soils, Dalton hypothesized that sorption of phenolic acids by soil, and the associated protection from microbial degradation, could permit the build-up of phenolic acids to phytotoxic concentrations in soils. The research described here summarizes tests of this hypothesis under laboratory conditions.

Microbial utilization of simple phenolic acids, such as ferulic and *p*-coumaric acid, leads to the production of other phenolic acids, such as vanillic acid, *p*-hydroxybenzoic acid, and/or protocatechuic acid before the aromatic ring structure is broken (Turner and Rice, 1975; Martin and Haider, 1976; Blum and Dalton, 1985; Blum and Shafer, 1988). Furthermore, the magnitude of plant responses to a phenolic acid mixture is not necessarily representative of the magnitude of response to any individual phenolic acid within that mixture (Einhellig et al., 1982; Williams and Hoagland, 1982; Blum et al., 1984, 1985b; Gerig and Blum, 1991; Blum, 1996). Thus, phytoxicity of a particular phenolic acid in soil may depend on the rate of microbial production and subsequent utilization of phenolic acid breakdown products. Here, we characterize the microbial production and utilization of initial phenolic acid breakdown products and discuss their potential importance in dose response bioassays.

METHODS AND MATERIALS

General Procedures. Cecil A_p and B_t horizon soil materials from the same soil profile (Typic Kanhaludults, clayey, kaolinitic, thermic) were obtained from the Piedmont of North Carolina, sieved (4-mm mesh), air-dried, and stored at room temperature in the laboratory (Dalton et al., 1987). Sieved (0.25-mm mesh) Cecil soil material was added to 15-ml Corex tubes (Corning Inc., Rochester, New York). Tubes containing 1 g of soil samples were capped (Bacti-Capall, Sherwood Medical Industries) and then autoclaved (15 min at 121°C) three times. Each autoclaving event was separated by one day. Initially, each tube of soil received 2 ml of a filter-sterilized (0.2-μm membrane filter) solution containing ferulic acid, ferulic acid + vanillic acid, *p*-coumaric acid, or ferulic

acid + *p*-coumaric acid (pH 5) of varied concentration. Tubes were then placed into a dark chamber (21–26°C) for 35 days. Each week the tubes were removed from the chamber and vortexed. On day 35, 0.5 ml full-strength Hoagland's solution without micronutrients (pH 5) (Hoagland and Arnon, 1950) and 0.5 ml soil extract containing microbes or 0.5 ml filter-sterilized soil extract (see below) were added. Soils were then extracted with water, EDTA (0.25 M, pH 7), or citrate (0.125 M, pH 7) at various intervals.

Soil Extracts for Infesting Soils with Microbes. Soil extracts used to infest autoclaved soil materials with microbes were obtained from a cup containing 150 g of a Cecil A_p or B_t horizon soil–sand mixture (1:2 by weight) treated with 5 ml ferulic acid, ferulic + vanillic acid, *p*-coumaric acid (0.5 mM each), or ferulic acid + *p*-coumaric acid (0.25 mM each), 10 ml full-strength Hoagland's solution without micronutrients, and 15 ml water. After four to five days in a dark incubator at 32°C, the soil–sand mixture was mixed with 300 ml water in a beaker. Soil particles were allowed to settle in the beaker before the resulting solution was decanted and filtered through Whatman No. 1 filter paper in a Buchner funnel. This extract was used to infest soil samples. A portion of this extract was also filter-sterilized (0.2-μm membrane filter) before addition to soil material to act as a sterile control.

Soil Extractions. Soil samples in the tubes were extracted with water or 0.25 M ethylenediamine tetraacetic acid (EDTA, pH 7) (Blum et al., 1994) or 0.125 M citrate, pH 7 (Blum, 1997) by adding 3 ml of water, 0.5 M EDTA, or 0.25 M citrate, respectively, to tubes containing 3 ml of solution (see above). Tubes were vortexed immediately after addition of the extractant. Tubes were stored in the dark for 2.5 hr and vortexed again just before centrifugation (10 min at 12,100*g*). Supernatants were filter-sterilized (0.2-μm membrane filter) for quantification of ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and/or vanillic acid by HPLC (Blum et al., 1994).

Recovery of phenolic acids by water extractions, the difference in recovery of phenolic acids between EDTA or citrate and water extractions, and the difference in recovery of phenolic acids between added and that recovered by EDTA or citrate extractions were used to characterize free (i.e., solution), reversibly sorbed, and fixed (unavailable) phenolic acids in soils, respectively. This differs from the traditional soil chemical approach of extracting each soil sample with water and then with EDTA or citrate. Since both methods gave essentially identical results, I used the more convenient method (Blum, unpublished data).

Specific Experimental Objectives. Experiments were designed with the following specific objectives: experiment 1—characterize, at steady state, the soil solution, reversibly sorbed and fixed fractions of ferulic acid for different concentrations of ferulic acid added to Cecil A_p and B_t horizon soil materials and characterize, in general, how soil solution and reversibly sorbed fractions change

once soil microbes are introduced into these steady state systems; experiment 2—characterize, in greater detail, how the actions of soil microbes modify soil solution and reversibly sorbed fractions of ferulic acid in Cecil A_p and B_t horizon soil materials; experiment 3—characterize microbial production of vanillic acid, an initial breakdown product of ferulic acid, when either ferulic acid alone or ferulic and vanillic acid are added to Cecil B_t horizon soil material, and characterize the microbial utilization of ferulic acid and vanillic acid under both conditions; and experiment 4—characterize the production of vanillic acid or *p*-hydroxybenzoic acid, the initial breakdown products of ferulic acid and *p*-coumaric acid, respectively, when ferulic acid, *p*-coumaric acid, or ferulic acid and *p*-coumaric acid are added to Cecil A_p and B_t horizon soil materials and characterize the microbial utilization of ferulic acid, vanillic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid in these systems.

Experimental Designs and Data Analyses. The treatment designs were as follows: Experiment 1—two soil materials (Cecil A_p and B_t horizon soil materials) × three concentrations of ferulic acid (1, 2, or 3 μmol/g) × presence or absence microbes × two extractants (water, EDTA) × two replicates × three extraction times (144 tubes); experiment 2—two soil materials from experiment 1 × one concentration of ferulic acid (2 μmol/g) × presence of microbes × two extractants (water, citrate) × four replicates × five extraction times (80 tubes); experiment 3—one soil material (Cecil B_t horizon) × two phenolic acid treatments (1 μmol/g ferulic or one μmol/g ferulic + 1 μmol/g vanillic acid) × presence of microbes × two extractants (water, citrate) × three replicates × eight extraction times (96 tubes); and experiment 4—two soil materials from experiment 1 × three phenolic acid treatments (1 μmol/g ferulic acid or *p*-coumaric acid, or 0.5 μmol/g of ferulic + 0.5 μmol/g *p*-coumaric acid) × presence of microbes × one extractant (citrate) × two replicates × nine extraction times (108 tubes). For experiment 4, some soil samples were also extracted at three time intervals with water (36 tubes). For all experiments, whenever soil samples no longer contained detectable levels of phenolic acids, extractions were discontinued. Data were analyzed by JMP, a statistical package from SAS Institute, Inc., utilizing linear and polynomial regressions and/or means comparisons. Significant differences between means were determined by the Tukey-Kramer HSD test for all pairs. A level of significance, α, of 0.05 was used.

RESULTS

Experiment 1—Phenolic Acid Concentrations. Recovery of ferulic acid by EDTA and water extractions from sterile soils was directly related to the concentration of ferulic acid added initially, was greater for EDTA than for water extractions, and was roughly equivalent for EDTA extractions of Cecil A_p and Cecil B_t horizon soil materials (Table 1, Figure 1). After 35 days, 29% ± 0.6%

TABLE 1. PARTIAL REGRESSION COEFFICIENT, P, AND r^2 VALUES FOR FERULIC ACID RECOVERED BY EDTA OR WATER IN CECIL A_p OR B_t HORIZON SOIL MATERIALS IN PRESENCE OR ABSENCE OF MICROBES

Extractant	Microbes	Soil	Conc (μmol/g soil)	Intercept	Linear	Quadratic	P	r^2
EDTA[a]	Absent	A		0.0082	0.7015		<0.001	0.99
		B		0.0590	0.6995		<0.001	0.99
Water[a]	Absent	A		−0.0336	0.6495		<0.001	0.99
		B		0.0910	0.3742		<0.001	0.99
EDTA[b]	Present	A	1	0.4506	−0.0065	0.00002	0.0024	0.99
			2	1.8383	−0.0305	0.00012	0.0472	0.95
			3	3.9830	−0.0673	0.00028	<0.001	0.99
		B	1	0.8460	−0.0052		<0.001	0.99
			2	1.6095	−0.0066		0.0043	0.95
			3	2.6331	−0.0115		0.0032	0.96
Water[b]	Present	A	1	0.2858	−0.0046	0.00002	0.2023 NS	0.80
			2	1.5989	−0.0271	0.00011	0.0086	0.99
			3	3.4089	−0.0589	0.00024	0.0157	0.98
		B	1	0.4186	−0.0023		0.0091	0.92
			2	0.9331	−0.0044		0.0053	0.95
			3	1.3602	−0.0049		0.0075	0.93

[a] Dependent values are μmol/g soil recovered; independent values are μmol/g added at the beginning.
[b] Dependent values are μmol/g soil recovered; independent values are time (48, 96, or 144 hr after introduction of microbes).

(mean ± SE) of the ferulic acid added was fixed and could not be recovered by EDTA extraction from Cecil A_p horizon soil material; 63% ± 0.3% was in a free form (soil solution) and could be recovered by either extractant; and 8% ± 0.6% was reversibly sorbed and could be recovered only by the EDTA extractant (Figure 1). For Cecil B_t horizon soil material these values were 26% ± 0.7%, 43% ± 0.6%, and 31% ± 0.6%, respectively. Reversible sorption of ferulic acid was thus four times greater for the B_t than the A_p horizon soil material. The proportion of fixed ferulic acid, however, was similar for both horizon soil materials. Since no significant differences in recovery of ferulic acid were observed for both extractants for days 37, 39, and 41 after initial addition of ferulic acid, it was concluded that the soil systems were essentially at steady state after 35 days. In the absence of microbes and just before addition of nutrient solution and soil extract (source of microbes), the ratios of reversibly sorbed to free (solution) ferulic acid for Cecil A_p and B_t horizon soil materials were 0.12 ± 0.01 and 0.72 ± 0.02, respectively.

Recovery of ferulic acid by EDTA and water in the presence of microbes declined in a curvilinear and linear manner for Cecil A_p and B_t horizon soil materials, respectively (Table 1). Since reversibly sorbed and/or solution ferulic acid recovered from a number of soil samples were zero and the number of data points for each concentration was small (Figure 2), meaningful trends for reversibly sorbed/soil solution ratios could not be calculated.

Experiment 2—Fixation, Reversible Sorption, and Microbial Utilization of Ferulic Acid. After 35 days and immediately after the addition of the nutrient solution and the soil extract (source of microbes), 18% ± 0.7% of the ferulic acid added could not be recovered (fixed) by citrate extraction from Cecil A_p horizon soil material, 69% ± 0.5% was in the free form (soil solution), and 13% ± 0.3% was reversibly sorbed. For Cecil B_t horizon soil material, these values were 16% ± 1%, 45% ± 0.9%, and 38% ± 1.9%, respectively. Reversible sorption of ferulic acid was thus three times greater for B_t than for A_p horizon soil material. The fixed ferulic acid was similar for both horizon soil materials. The r/s ratios for Cecil A_p and B_t horizon soil materials were 0.19 ± 0.005 and 0.85 ± 0.06, respectively.

Once microbes became active, the r/s ratio increased in a curvilinear manner with time for both horizon soil materials, but the increase was much more rapid for the A_p than the B_t horizon soil material (Figures 3 and 4). The differences in the patterns of microbial utilization of free and reversibly sorbed ferulic acid were characterized by rescaling the amount recovered for each time period by log transformation (Figure 5). After microbial activity had stabilized (24 hr after introduction of microbes), the declines of the ln-transformed solution ferulic acid for both horizon soil materials and the ln-transformed reversibly sorbed for Cecil A_p horizon soil material were linear (first-order reaction). The decline

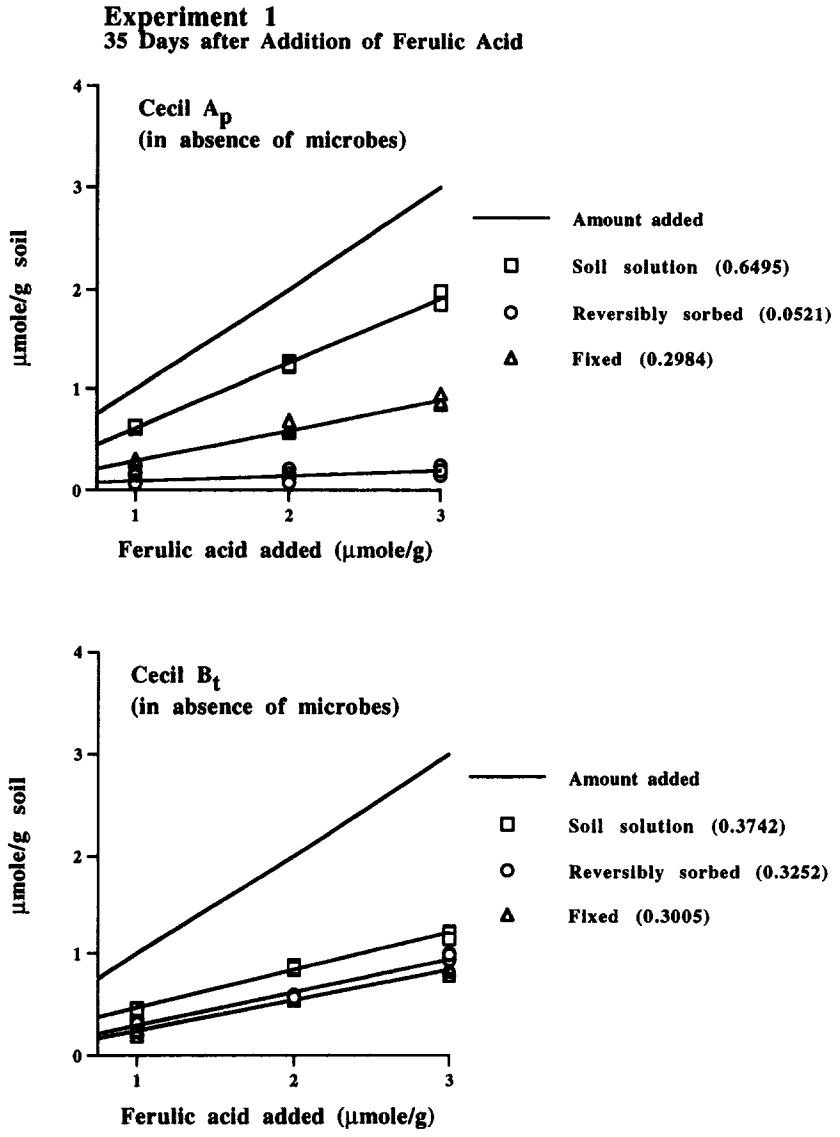


FIG. 1. Amount of ferulic acid in soil solution, reversibly sorbed and fixed in noninfested Cecil A_p and B_t horizon soil materials in the absence of microbes (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean. Values in parentheses are slopes.

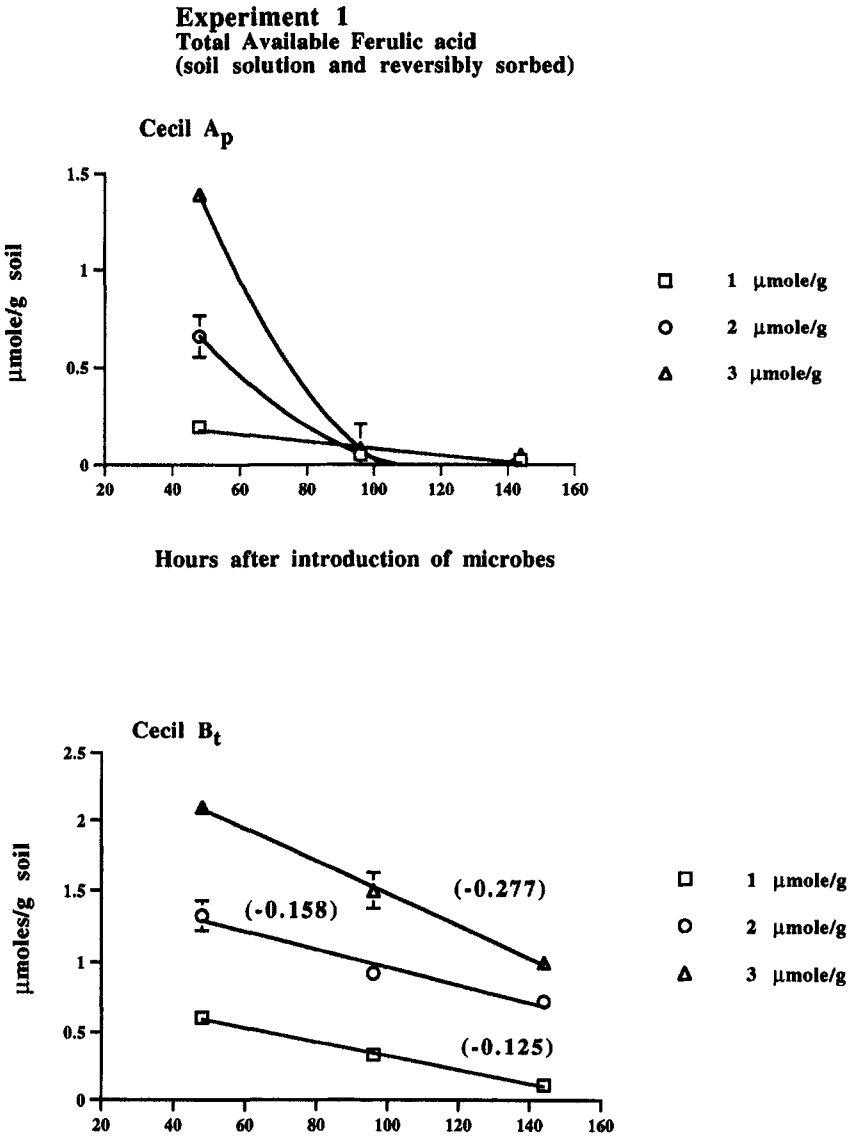


FIG. 2. Microbial utilization of available (solution and reversibly sorbed) ferulic acid (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean. Values in parentheses are slopes.

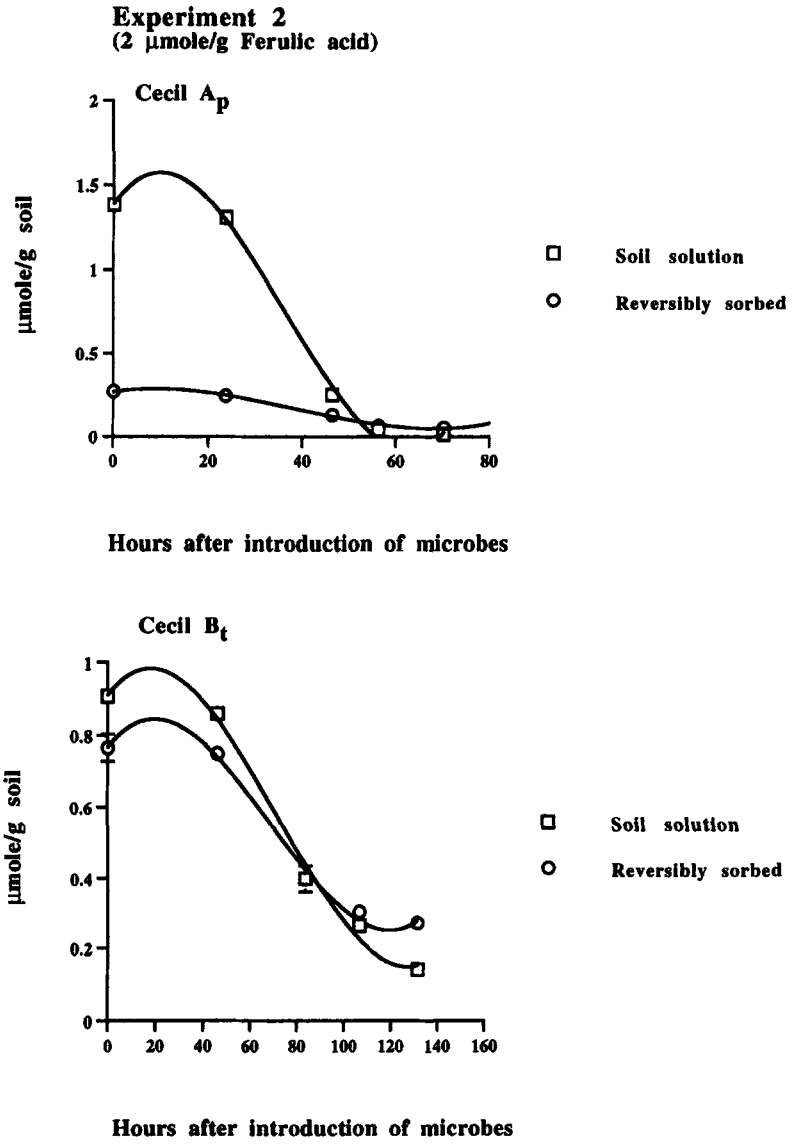


FIG. 3. Microbial utilization of ferulic acid in soil solution and reversibly sorbed to Cecil A_p and B_t horizon soil materials (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

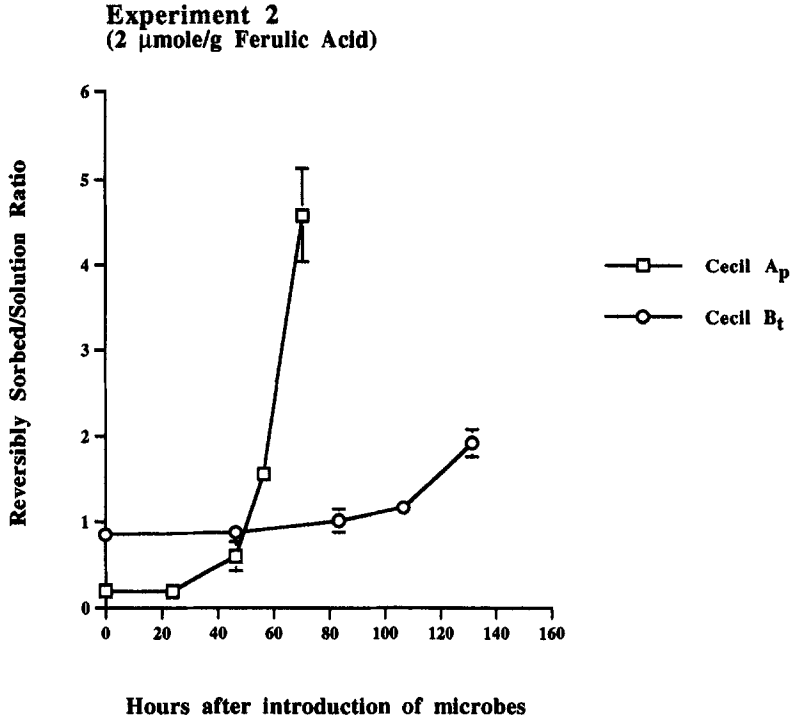


FIG. 4. Changes in reversibly sorbed/solution ratio as ferulic acid is utilized by microbes in Cecil A_p and B_t horizon soil materials (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

of the ln-transformed reversibly sorbed ferulic acid for Cecil B_t horizon soil material, however, was multiphasic (Figure 5). The multiphase pattern for the 2 μmol ferulic acid treatment suggested that reversibly sorbed ferulic acid for Cecil B_t horizon material consisted of ferulic acid fractions with different rate constants for desorption.

Experiment 3—Ferulic Acid and Its Initial Breakdown Product. Soil solution and reversibly sorbed ferulic acid in Cecil B_t horizon soil material was used at roughly the same rates by microbes (Figure 6). After microbial activity stabilized, use of soil solution and reversibly sorbed (1 μmol/g treatment) ferulic acid could be described by first-order reactions, which differed from the complex response observed for the 2 μmol/g treatment (Figure 5). Concentrations of vanillic acid, a microbial breakdown product of ferulic acid, increased for a period of time and then decreased as ferulic acid became depleted in the Cecil B_t horizon soil system (Figure 6). Maximum concentrations of the solution (0.23

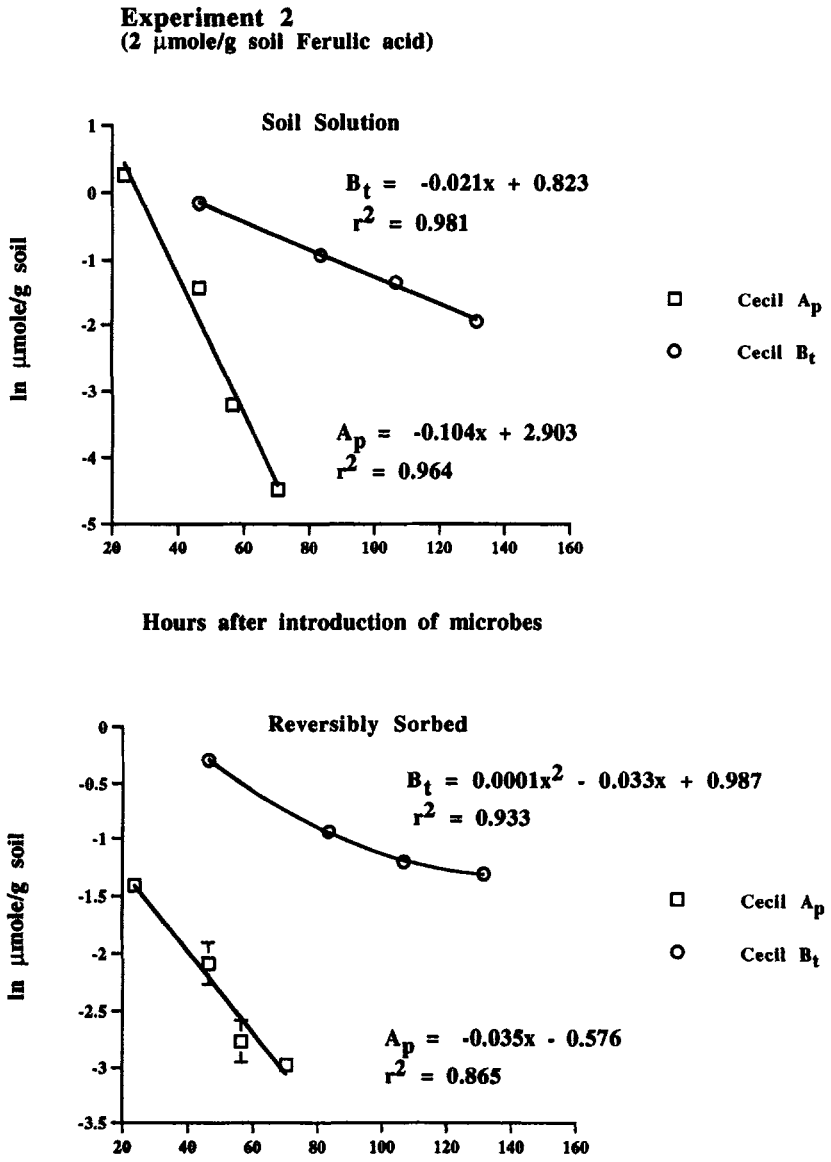


FIG. 5. Kinetics of soil solution and reversibly sorbed ferulic acid in Cecil A_p and B_t horizon soil materials (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

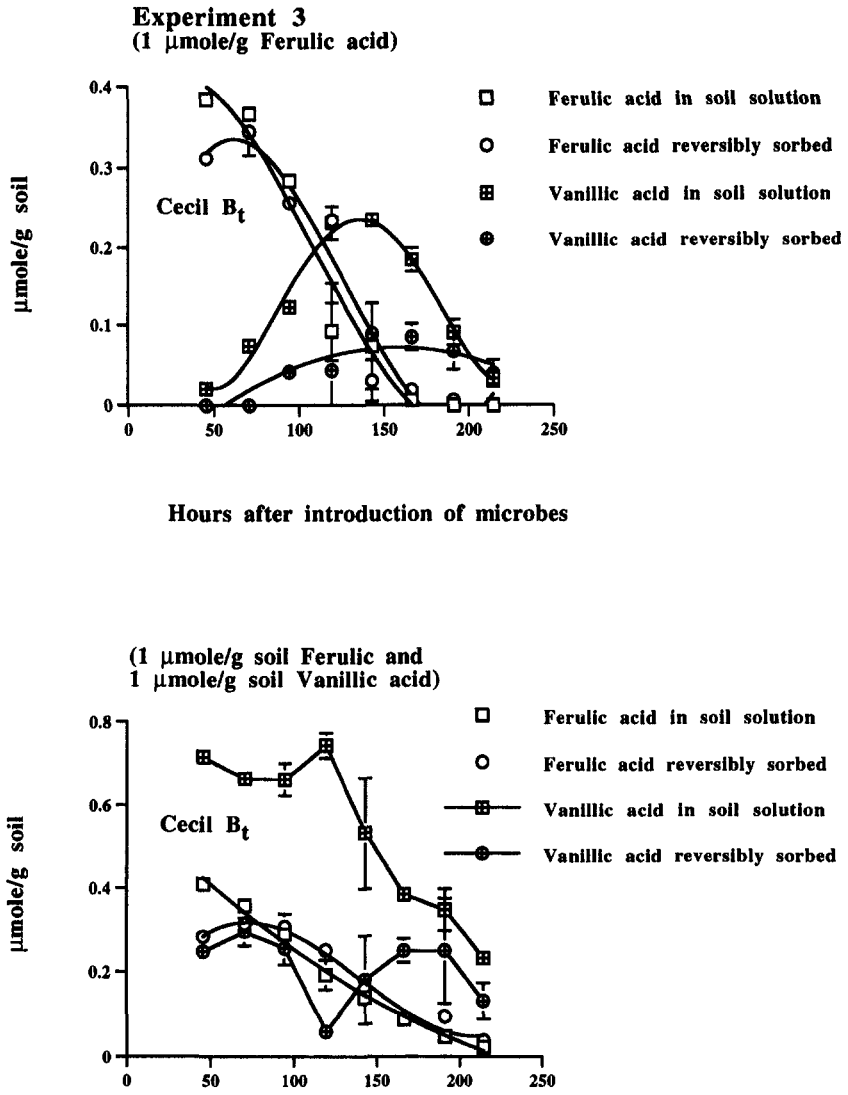


FIG. 6. Microbial utilization of soil solution and reversibly sorbed ferulic acid in the presence and absence of exogenously supplied vanillic acid in Cecil B_t horizon soil materials. Microbial production and utilization of soil solution and reversibly sorbed vanillic acid in Cecil B_t horizon soil materials (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

TABLE 2. PARTIAL REGRESSION COEFFICIENTS AND r^2 VALUES FOR TOTAL AVAILABLE (FREE AND REVERSIBLY SORBED) PHENOLIC ACIDS RECOVERED FROM CECIL HORIZON SOIL MATERIALS IN PRESENCE OF MICROBES[a]

Treatment	Soil	Intercept	Linear	Quadratic	r^2
Experiment 3					
1 μmol/g ferulic acid	B_t	0.1750	1.8424	−1.4732	0.94
Experiment 4					
1 μmol/g ferulic acid	A_p	0.0452	0.9662		0.99
1 μmol/g *p*-coumaric acid	A_p	0.0458	0.9936		0.99
0.5 μmol/g ferulic acid and 0.5 μmol/g *p*-coumaric acid	A_p	−0.0346	0.9819		0.99
1 μmol/g ferulic acid	B_t	0.0079	2.0374	−1.4149	0.99
1 μmol/g *p*-coumaric acid	B_t	0.0123	2.4592	−1.1717	0.99
0.5 μmol/g ferulic acid and 0.5 μmol/g *p*-coumaric acid	B_t	−0.0950	−0.0196	1.3793	0.99

[a]Dependent values are μmol/g soil recovered for treatment phenolic acid(s); independent values are sums of μmol/g soil recovered for treatment phenolic acid(s) and the initial microbial breakdown product(s). All regressions are statistically significant.

± 0.003 μmol/g) and reversibly sorbed (0.09 ± 0.006 μmol/g) vanillic acid were observed 143 hr after the introduction of microbes. The relationship between total available (free and reversibly sorbed) ferulic acid recovered over time, and the sum of total available ferulic and vanillic acid recovered over time was curvilinear (Table 2).

When both ferulic acid and vanillic acid (1 μmol/g each) were added to the Cecil B_t horizon soil system, microbial utilization of ferulic acid was lower than when only ferulic acid was added (Figure 7). Soil solution vanillic acid concentrations initially declined slightly, increased to a peak, and then declined very rapidly (Figure 6). Reversibly sorbed vanillic acid concentrations, on the other hand, increased slightly, decreased, increased to approximately the previous level, and then decreased again. The reversibly sorbed/soil solution ratio for the combined data (i.e., ferulic acid and ferulic acid + vanillic acid) increased in a linear manner over time for the Cecil B_t horizon soil material as ferulic acid (slope = 0.009, r^2 = 0.13) or vanillic acid (slope = 0.004, r^2 = 0.26) was used by microbes.

Experiment 4—Ferulic Acid and/or p-*Coumaric Acid and Their Initial Breakdown Products.* After 35 days and immediately after the addition of nutrient solution and the soil extracts (source of microbes), 29% of ferulic acid and 21% of *p*-coumaric acid were fixed and could not be recovered by citrate from Cecil A_p horizon soil material, 57–67% of ferulic acid or *p*-coumaric acid were in the

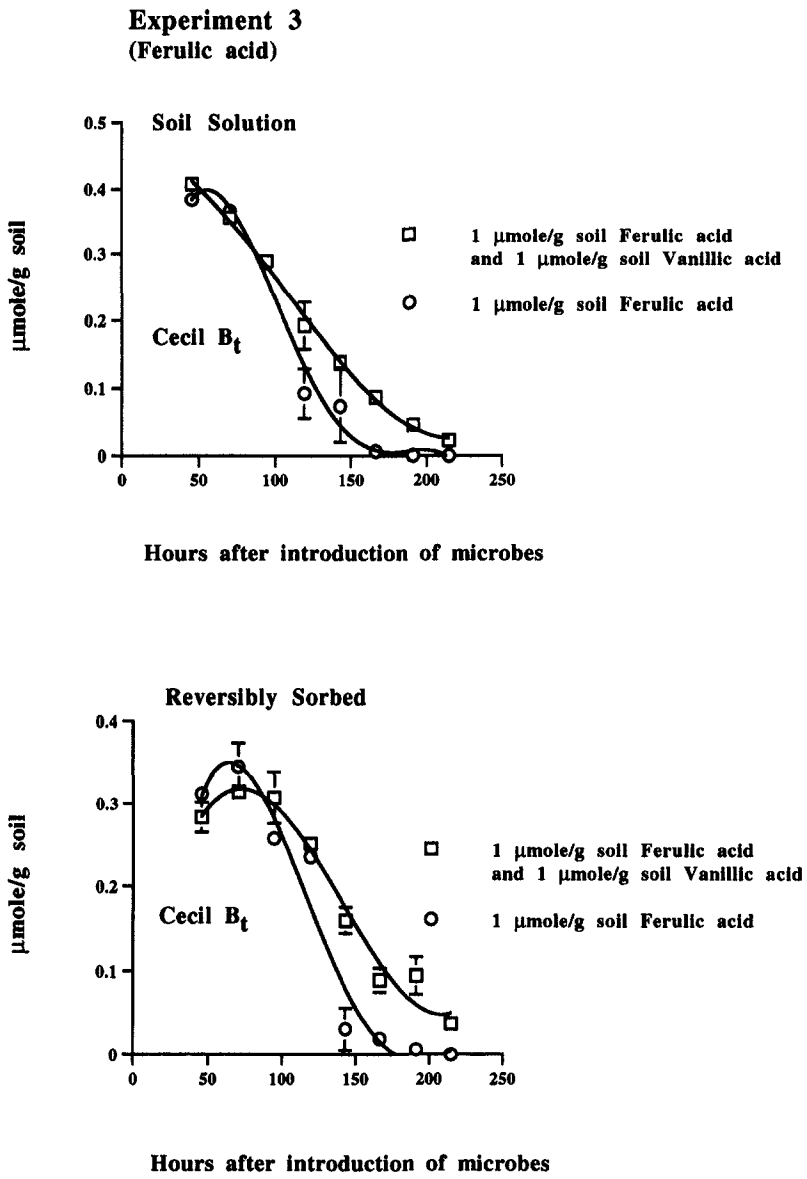


FIG. 7. Comparison of soil solution and reversibly sorbed ferulic acid in the presence and absence of exogenously supplied vanillic acid in Cecil B_t horizon soil material (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

free form (solution), and 10–14% of ferulic acid or *p*-coumaric acid were reversibly sorbed (Table 3). For Cecil B_t horizon soil material, the fixed values were 25% for ferulic acid and 13% for *p*-coumaric acid, and 42–52% and 32–35% for the free and reversibly sorbed, respectively. The reversibly sorbed/solution ratios for ferulic acid or *p*-coumaric acid were 0.2 and 0.72 for Cecil A_p and B_t horizon soil materials, respectively.

One hundred eight hours after the introduction of microbes, the reversibly sorbed/solution ratios for ferulic acid, p-coumaric acid, and vanillic acid were 1.5–2 for Cecil B_t horizon soil material. Ratios were not determined for A_p horizon soil material. Total available (i.e., free and reversibly sorbed) ferulic acid and/or *p*-coumaric acid and their initial breakdown products, vanillic acid and *p*-hydroxybenzoic acid, were readily utilized by microbial populations (Figures 8–10). However, there was one exception. Vanillic acid in B_t horizon soil material treated with both ferulic acid and *p*-coumaric acid was not readily metabolized (Figure 10). Maximum concentration of vanillic acid (14.5 ± 2.5 and 5.0 ± 0.0 μmol/g soil for ferulic and ferulic + *p*-coumaric treatments, respectively) or *p*-hydroxybenzoic acid (11.0 ± 0.0 and 8.0 ± 1.0 μmol/g soil for ferulic and ferulic + *p*-coumaric treatments, respectively) in Cecil A_p horizon soil material was observed 48 hr after introduction of microbes. Maximum concentrations of vanillic acid (34.5 ± 6.5 and 47.5 ± 0.5 μmol/g soil for ferulic and ferulic + *p*-coumaric treatments, respectively) and *p*-hydroxybenzoic acid (32.0 ± 0.0 and 7.5 ± 0.5 μmol/g soil for ferulic and ferulic + *p*-coumaric treatments, respectively) in Cecil B_t horizon soil material were observed 71 hr after introduction of microbes for all but the 47.5 μmol/g soil vanillic acid value, which occurred at 108 hr. The relationship between total available (free and reversibly sorbed) ferulic acid and/or *p*-coumaric acid recovered over time, as well as the sum of various combinations of total available ferulic acid, vanillic acid, *p*-coumaric acid, and *p*-hydroxybenzoic acid recovered over time were linear for Cecil A_p horizon soil material and curvilinear for Cecil B_t horizon soil material (Table 2).

DISCUSSION

The pK value for simple phenolic acids (e.g., ferulic acid, vanillic acid) is approximately 4.5; thus, both anionic and protonated (nonionic) forms are present in most soil solutions. The anionic forms could bind to positively charged sites (e.g., Al and Fe oxides) on soil surfaces (Watson et al., 1973) and indirectly to negatively charged sites by way of multivalent cation bridges (Greenland, 1965, 1971). The protonated forms can be sorbed by soil organic matter (Chiou, 1989; Hasset and Banwart, 1989) and/or polymerized into humic substances in the soil (Martin et al., 1972; Martin and Haider, 1976; Haider et al.,

TABLE 3. PARTITIONING OF FERULIC ACID AND/OR *p*-COUMARIC ACID IN CECIL A_p OR B_t HORIZON SOIL AFTER 35 DAYS IN ABSENCE OF MICROBES[a]

Phenolic acid	Soil	Initial concentration (μmol/g)	Solution (%)	Reversibly sorbed (%)	Fixed (%)	Reversibly sorbed/ solution ratio
Ferulic acid	A_p	1	58 ± 1	13 ± 2	28 ± 0	0.23 ± 0.02
p-Coumaric acid	A_p	1	67 ± 1	14 ± 1	19 ± 1	0.21 ± 0.02
Ferulic acid and		0.5	57 ± 1	13 ± 2	30 ± 1	0.22 ± 0.03
p-coumaric acid	A_p	0.5	67 ± 0	10 ± 3	23 ± 3	0.15 ± 0.04
Ferulic acid	B_t	1	42 ± 2	32 ± 1	26 ± 1	0.75 ± 0.05
p-Coumaric acid	B_t	1	51 ± 1	35 ± 1	13 ± 0	0.70 ± 0.02
Ferulic acid and		0.5	43 ± 1	32 ± 0	25 ± 1	0.75 ± 0.01
p-coumaric acid	B_t	0.5	52 ± 1	35 ± 1	13 ± 1	0.68 ± 0.03

[a]Only soil type was significantly different for solution, reversibly sorbed, and reversibly sorbed/solution ratio; only phenolic acid was significantly different for fixed (not recoverable by citrate).

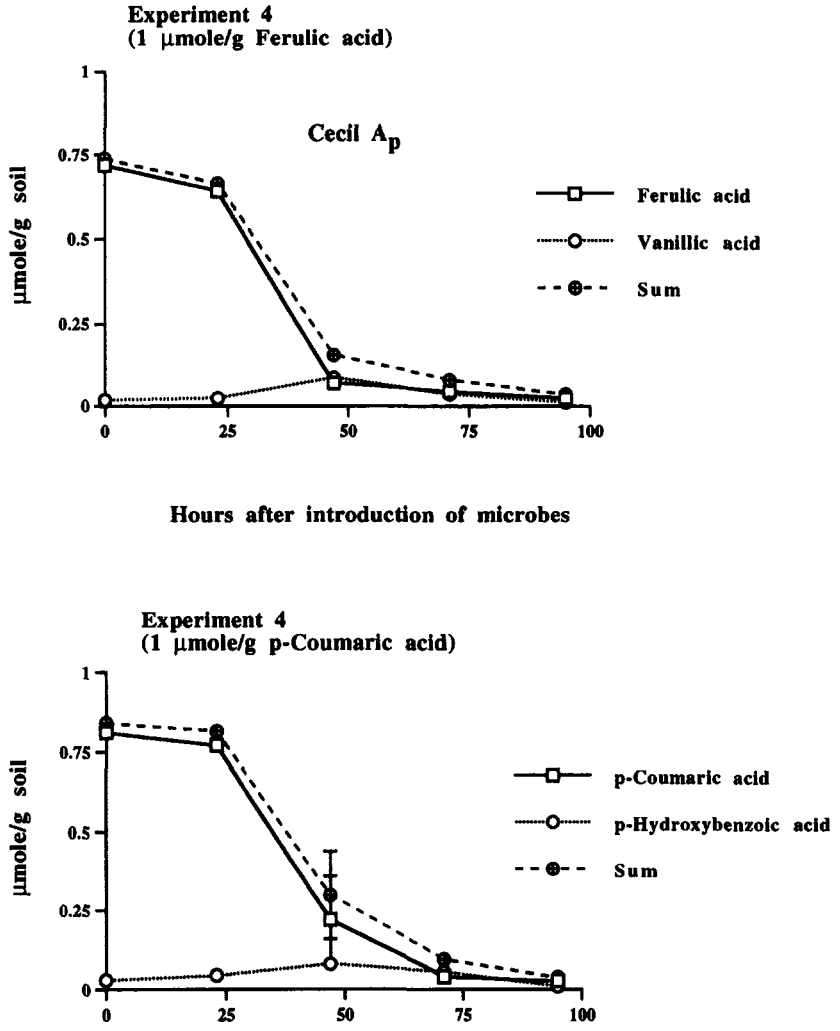


FIG. 8. Microbial utilization of available ferulic acid and *p*-coumaric acid, production of and utilization of available breakdown products of ferulic acid (i.e., vanillic acid) and *p*-coumaric acid (i.e., *p*-hydroxybenzoic acid), and the sum of each phenolic acid and its breakdown product in Cecil A_p horizon soil material (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

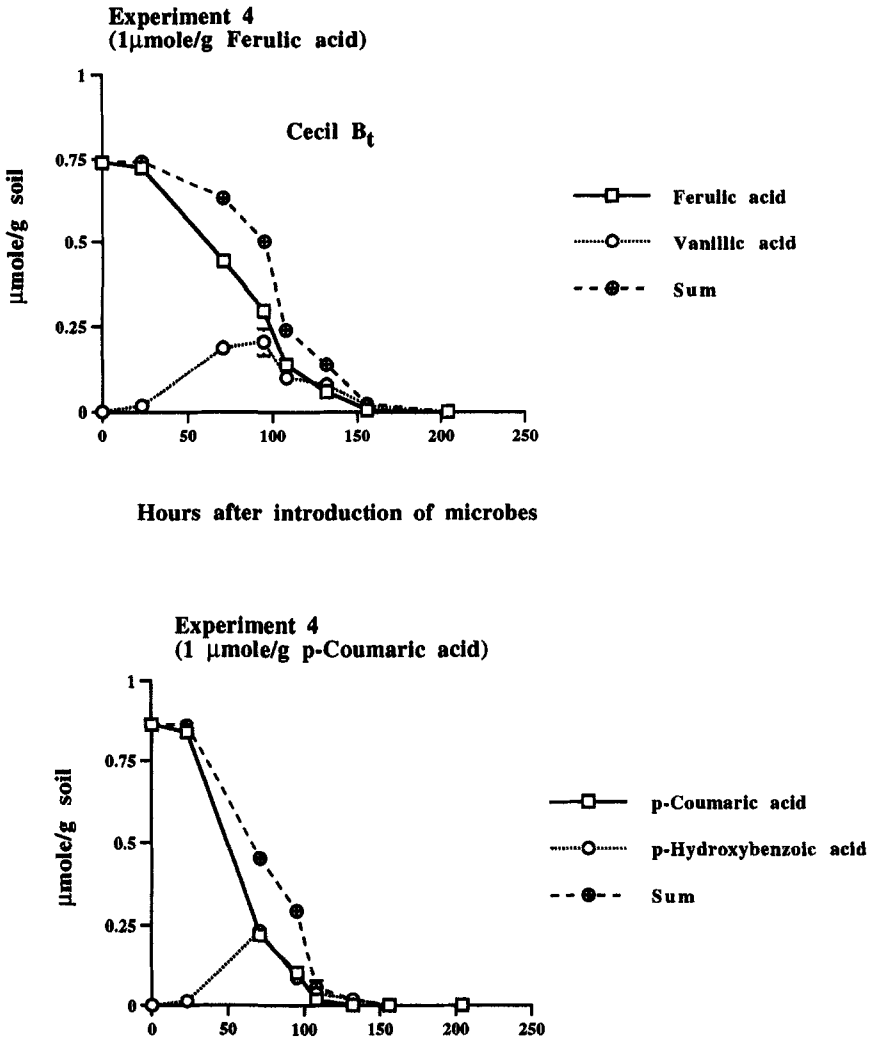
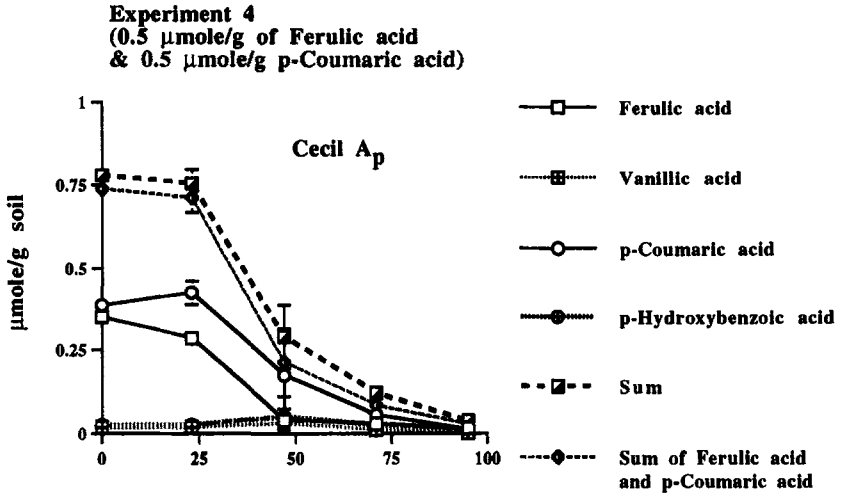


FIG. 9. Microbial utilization of available ferulic acid or *p*-coumaric acid, production of and utilization of available breakdown products of ferulic acid (i.e., vanillic acid) or *p*-coumaric acid (i.e., *p*-hydroxybenzoic acid), and the sum of each phenolic acid and its breakdown product in Cecil B_t horizon soil material (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.



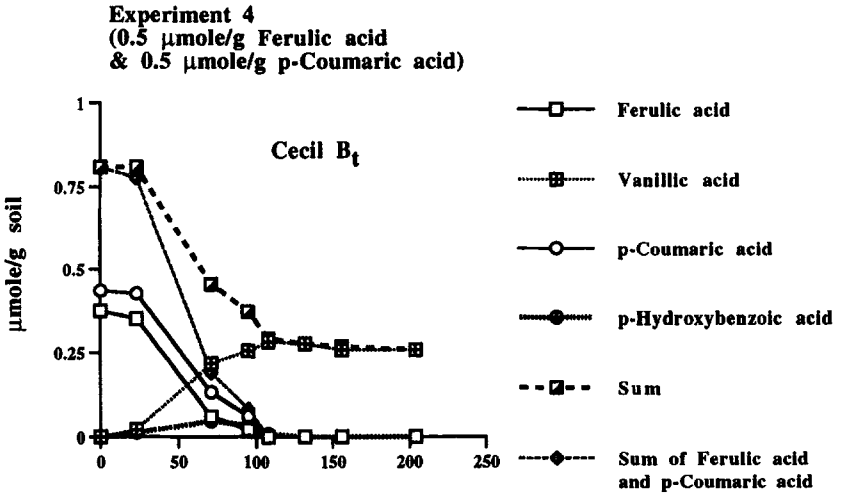


FIG. 10. Microbial utilization of available ferulic acid and *p*-coumaric acid, production of and utilization of available breakdown products of ferulic acid (i.e., vanillic acid) and *p*-coumaric acid (i.e., *p*-hydroxybenzoic acid), the sum of ferulic and *p*-coumaric acid and their breakdown products, and the sum of ferulic acid and *p*-coumaric acid in Cecil B_t horizon soil material (means ± standard errors). Absence of error bars indicates that the error bars are smaller than the symbol representing the mean.

1977; Wang et al., 1986). Sorption may also occur through hydrogen bonding and van der Waal's forces (Greenland, 1965, 1971). The binding strengths of the resulting complexes vary considerably. Some complexes (e.g., those resulting from hydrogen bonding, van der Waal's forces, anion exchange or cation bridging) are rather easily disrupted (i.e., desorbed), while others (e.g., those resulting from ligand exchange, oxidative reactions with mineral surfaces, polymerization) are fixed into recalcitrant organic matter or bound on clays and thus are essentially unavailable. The solution and reversibly sorbed fractions comprise the total phenolic acid pool available for allelopathic interactions, and the losses due to the fixed fraction reduce the size of this available pool.

A major concern regarding allelopathic interactions that involve phenolic acids pertains to the fact that concentrations of individual phenolic acids, including ferrulic acid and *p*-coumaric acid, recoverable from field soils are well below concentrations required for inhibition of germination and seedling growth (Blum, 1996). Since most phenolic acids in soil solutions have a short half-life, it has been suggested that reversible sorption of phenolic acids may provide some protection against microbial degradation and that such protection may allow a build-up of phenolic acids in soils to phytotoxic levels (Dalton, 1989). The data presented here for ferulic acid and *p*-coumaric acid suggest that reversibly bounded phenolic acids in Cecil horizon soils are not sufficiently protected from microbial utilization to allow for the accumulation of these phenolic acids to phytotoxic levels. In fact, reversibly sorbed phenolic acids, such as ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid and vanillic acid, were readily utilized by microbes, although such utilization occurs at slightly slower rates than for free (solution) phenolic acids. The release of reversibly sorbed phenolic acids from Cecil horizon soil materials, at concentrations below 1 μmol/g, could be described by first-order reactions, i.e., release was closely related to the microbial depletion of the phenolic acid in the soil solution.

The phytotoxicity of individual phenolic acids, alone or in mixtures, varies with soil type, pH, mineral nutrition, and the presence of other phenolic acids or alternative carbon sources (Hall et al., 1982, 1983; Blum et al., 1985a; Blum, 1995; Pue et al., 1995). The observed microbial production of vanillic acid and *p*-hydroxybenzoic acid from ferulic acid and *p*-coumaric acid, respectively, by soil microbes and their differential soil fixation and microbial utilization provided an excellent opportunity to characterize how the microbial utilization of an original phenolic acid added to the Cecil horizon soil systems related to the microbial production and utilization of its initial breakdown product. Such information is important in interpreting phenolic acid dose–response bioassays.

Although microbial populations were not determined for these experiments, previous microbial population estimates for similarly treated soil samples demonstrated that microbial populations were much greater in A_p than in B_t horizon

soil materials (Blum and Shafer, 1988). In addition, pretreatment of Cecil horizon soil materials with phenolic acids, as was done here, leads to natural selection and/or induction of microorganisms that are capable of utilizing phenolic acids as sole carbon sources (Blum and Shafer, 1988; Flint, Shafer, and Blum, unpublished data). This might happen in natural soils during periods of phenolic acid production and/or input by root exudates or debris leachates. However, bacterial isolates from phenolic acid-pretreated microbial communities vary in their ability to utilize other phenolic acids (Flint, Shafer, and Blum, unpublished data). Thus, as expected, microbial utilization of the phenolic acids added to the soil systems was rapid in both soil systems, but much more rapid in Cecil A_p than in Cecil B_t horizon soil materials. Initial utilization of breakdown products was delayed. The delay was longer in Cecil B_t than in Cecil A_p horizon soil systems.

The extent of the delay in assimilation of the initial breakdown products and the reduced utilization of ferulic acid when both ferulic acid and vanillic acid were added in the Cecil B_t horizon soil systems suggested preferential utilization of phenolic acids. Preferential microbial utilization of one carbon source over another has been shown in batch culture (Harder and Dijkhuizen, 1982; Papanastasiou, 1982) and in plant–microbe–soil systems (Pue et al., 1995). In pure cultures, sequential utilization in carbon sources frequently leads to diauxic growth of the bacteria. Diauxic growth is characterized by two exponential phases of growth separated by a phase of no net increase in microbial biomass, an indication that different carbon sources are being used during the two growth phases. In multispecies microbial communities, evidence of diauxic growth is unusual because different species of microbes have different carbon resource preferences. Nonetheless, the earlier and more rapid utilization of glucose, phenylalanine, or *p*-hydroxybenzoic acid over *p*-coumaric acid observed in plant–microbe–soil systems (Pue et al., 1995) and the reduced rate of ferulic acid utilization when both ferulic and vanillic acid were added to Cecil soil suggested that such sequential carbon utilization does occur in multispecies systems. Additional evidence for variation in carbon utilization in soils has been provided by Haider and Martin (1975), who observed that mineralization of ^{14}C-labeled ring carbon from glucose acid was more rapid than from phenolic acids, and Sugai and Schimel (1993), who observed in taiga soils from a series of successional stages that glucose, *p*-hydroxybenzoic acid, and salicyclic acid were processed very differently by soil microorganisms. More than twice as much glucose was converted to biomass than either of the phenolic acids, and although both phenolic acids were metabolized, only *p*-hydroxybenzoic acid was assimilated by microbes.

Effects of phytotoxic phenolic acids on plant growth are a function in part of phenolic acid concentrations (Blum et al., 1985b). Studies of mixtures of

phenolic acids in laboratory bioassays indicate that plant responses to a single phenolic acid are not necessarily representative of the responses to mixtures (Gerig and Blum, 1991; Blum, 1996). Specifically, as the number of phenolic acids added to soil increased, concentrations of the individual phenolic acids required to bring about a growth inhibition declined (Blum, 1996). Thus, in bioassays, the microbial breakdown products and their subsequent utilization could substantially influence the outcome of phenolic acid dose–response studies. In the Cecil A_p horizon soil system this appeared to be a minor concern, since microbial utilization of the substrates (ferulic acid and/or *p*-coumaric acid) and the synthesis and utilization of the breakdown products (vanillic acid and *p*-hydroxybenzoic acid) were closely balanced, as indicated by a linear slope of nearly 1 for the substrate and the sum of the substrate and product. For the Cecil B_t horizon soil system, however, the relationship between the substrate and the sum of the substrate and product over time was curvilinear, suggesting a potential for confounding of dose responses. This was particularly evident for the soil system supplied with both ferulic acid and *p*-coumaric acid. Both ferulic acid and *p*-coumaric acid were no longer detectable after 108 hr, but vanillic acid, a breakdown product of ferulic acid, was still detectable at 0.26 μmol/g soil after 204 hr. Since growth inhibition of phenolic acid is rapidly lost once phenolic acids are removed from the root environment (Blum and Rebbeck, 1989), growth responses in such a system would be a product of ferulic acid, *p*-coumaric acid, and vanillic acid. The role of *p*-hydroxybenzoic acid, a breakdown product of *p*-coumaric acid, appeared to be of little consequence in this instance. The relative potency values of these phenolic acids on leaf expansion of cucumber seedlings grown in nutrient culture (Blum et al., 1985b) were 1.0 for ferulic acid, 0.86 for *p*-coumaric acid, 0.73 for vanillic acid, and 0.50 for *p*-hydroxybenzoic acid. The relative potencies of these phenolic acids were quantitatively less, but in the same order, for leaf expansion of cucumber seedlings grown in Portsmouth B_t horizon soil materials (Gerig and Blum, 1991).

In summary, the data presented here for ferulic acid and *p*-coumaric acid suggest that reversibly bound phenolic acids in Cecil horizon soils are not protected sufficiently from microbial utilization to allow for accumulation of these phenolic acids to phytotoxic levels. Differential soil fixation, microbial production of benzoic acids (e.g., vanillic acid and *p*-hydroxybenzoic acid) from cinnamic acids (e.g., ferulic acid and *p*-coumaric acid, respectively), and the resulting differential utilization of cinnamic and benzoic acids by soil microbes indicate that these processes can substantially influence the magnitude and duration of the phytotoxicity of individual phenolic acids in soils.

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EVIDENCE FOR A HOST-MARKING PHEROMONE IN WHITE SPRUCE CONE FLY, *Strobilomyia neanthracina*

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Abstract—Cone flies (*Strobilomyia* spp.) lay eggs on coniferous cones, and larvae must complete development within the cone on which the eggs are laid. Previous field surveys showed that egg distributions of several species are uniform, suggesting that females avoid ovipositing on cones with conspecific eggs or larvae. In both the field and laboratory, *S. neanthracina* females walked around cones following oviposition, touching their mouthparts to the cone as they walked. In laboratory bioassays, where females were presented with a cone on which they or another female had oviposited and touched with mouthparts, or one without an egg, females laid preferentially on cones without eggs. However, females laid randomly when presented with a cone on which a female had oviposited but been prevented from touching with her mouthparts following oviposition or another cone without an egg. This indicates that females deposit a host-marking pheromone with their mouthparts following oviposition and that this deters further oviposition on marked hosts. Laboratory and field bioassays indicated that the host-marking pheromone is located in the head and thorax of female flies.

Key Words—Marking pheromones, cone maggots, cone flies, *Strobilomyia neanthracina*, spruce, foraging behavior.

INTRODUCTION

In many insect species, such as leaf miners and fruit and seed feeders, offspring must complete development in the host on which the egg is laid. When food

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resources within a discrete host are relatively small, compared to the requirements of individual juveniles, survival or the size of survivors may be reduced if females oviposit on small hosts or hosts containing conspecifics (Mitchell, 1975; Quiring and McNeil, 1984a). For example, the fitness of larvae of several leaf miners (Murai, 1974; Stiling et al., 1984; Quiring and McNeil, 1984a; Bultman and Faeth, 1986; Auerbach, 1991), fleshy fruit feeders (Prokopy and Koyama, 1982; Averill and Prokopy, 1987a), and seed eaters (Mitchell, 1975) is decreased when more than one individual develops in the same host. In such systems, where the ovipositing female determines the quality of the environment of her offspring, there should be strong selection pressure to select hosts best suited for development of offspring (Jaenike, 1978; Quiring and McNeil, 1987; Thompson, 1988; but see Larsson and Ekbom, 1995).

In many systems, this strong selection pressure apparently has led to evolution of host-marking pheromones (HMP), which are deposited by females during or following oviposition (Roitberg and Prokopy, 1987; Roitberg and Mangel, 1988). Females encountering HMP usually reject marked hosts if unmarked hosts are readily available. By rejecting marked hosts, marking females reduce the probability that their offspring will be subjected to intraspecific competition with siblings or other conspecifics (or congenerics). Marking pheromones have been reported for over 30 species of phytophagous insects (Roitberg and Prokopy, 1987; Kouloussis and Katsoyannos, 1991; Rika, 1994).

Several weevils (Oshima et al., 1973; Messina and Renwick, 1985; Szentèsi, 1981) and flies (Zimmerman, 1979; Lalonde and Roitberg, 1992), whose offspring develop within seeds of their herbaceous hosts, have been reported to deposit HMP after ovipositing. Miller and Borden (1984) showed that females of the Douglas-fir cone gall midge, *Contarinia oregonensis* Foote, made fewer total visits and a greater proportion of exploratory visits (< 1 min in duration) on conelets that already contained midge eggs than on unoccupied conelets; they suggested that the midge may deposit an oviposition deterrent. However, except for the almond seed wasp (Kouloussis and Katsoyannos, 1991), there are no reports of HMP used by phytophagous insects of tree seeds and none for insects feeding on seed cones.

Cone flies (*Strobilomyia* spp.) lay eggs on coniferous cones, and larvae must complete development within the cone on which the egg is laid. Recent studies indicate that female cone flies are selective when choosing oviposition sites. For example, female larch cone flies, *Strobilomyia laricis* Michelsen and *S. viaria* (Huckett), as well as *S. neanthracina* Michelsen and *S. appalachensis* Michelsen (cone flies on white and black spruce, respectively), lay eggs preferentially on the largest cones, which contain the largest seeds and in which larvae obtain the highest larval weights (McClure, 1995; McClure et al., 1996; Sweeney and Quiring, unpublished data; Quiring et al., unpublished data; Fidgen et al., unpublished data). In addition, field surveys showed that egg distributions

of the two larch and the two spruce cone flies are uniform, suggesting that females avoid ovipositing on cones with cone maggot eggs or larvae (Sweeney and Turgeon, 1994; McClure, 1995; Fidgen et al., unpublished data; Sweeney, unpublished data).

In this paper we present evidence that cone flies deposit a HMP following oviposition that deters further oviposition by the same or other females. We carried out laboratory and field bioassays with *S. neanthracina*: (1) to determine if cone maggot females avoid ovipositing on cones with eggs, (2) to evaluate the mechanism responsible for discrimination of occupied cones, and (3) to determine in which body region the compound(s) responsible for marking hosts is contained.

METHODS AND MATERIALS

Study Sites. Field studies were carried out on interior spruce, a hybrid of white, *Picea glauca*, and Engelmann, *P. engelmannii* Parry, spruce at Skimikin Seed Orchard (50°47′N, 119°20′W), British Columbia, and on white spruce at Queensbury Seed Orchard (45°59′N, 67°7′W), near Fredericton, New Brunswick, Canada. Interior spruce trees were approximately 10 m high, 30 years old, and planted in a 5- × 5-m spacing at Skimikin. White spruce trees were approximately 1.5 m high, 10 years old, and planted in a 3- × 6-m spacing at Queensbury. Branches were enclosed in fine mesh sleeve cages at both Queensbury and Skimikin prior to oviposition to obtain egg-free cones for bioassays. Cone-bearing branches were collected when cones were receptive to cone flies (scales closed and cones turning down) and stored at 3°C with the ends of the shoots immersed in water until used in bioassays. Laboratory experiments were carried out under artificial lighting at 24 ± 2°C at Fredericton and Skimikin.

Behaviors Associated with Oviposition. Flies were observed in the field and during laboratory bioassays to identify preoviposition, oviposition, and postoviposition behaviors and their duration.

Influence of Eggs and Postoviposition Behavior. Laboratory bioassays were carried out in 1995 and 1996 to determine whether females avoided ovipositing in cones with eggs. Females used in bioassays in 1995 at Fredericton were collected with an aspirator from cones at Queensbury in 1995 and transferred to 37- × 28- × 29-cm screen cages provided with fresh cones, water, and an ad libitum diet. The diet was composed of 1.0 g agar, 100 ml water, 20.0 g sucrose, 50 g honey, and 17.5 g enzymatic yeast hydrolysate. Cages were kept in a shaded outdoor insectary. Each female was only used once in an experiment.

Females used in bioassays at Shimikin in 1995 and 1996 were collected as prepupae at either Skimikin, Queensbury, or Pokiok (46°7′N, 67°15′W) the previous year. Similarly, females used in bioassays in Fredericton in 1996 were

obtained as prepupae from Queensbury in 1995. To obtain pupae, we collected cones in late June, when fully developed larvae (i.e., prepupae) had begun to drop from cones to the ground. Cones from Skimikin were placed in burlap sacks above a plastic sheet, and prepupae that had exited cones and crawled onto the sheet were collected and placed on moistened paper towels where they pupated. Prepupae from Pokiok and Queensbury emerged when cones were submerged in water and were transferred to moist potting soil where they pupated.

Pupae from Queensbury and Pokiok were reared in a controlled environment chamber with relative humidity set at 70% and the potting soil moistened regularly. The light regime was 14L:10D during July and August, and the photophase gradually was reduced in the fall. In 1994, temperatures were held at a constant 18°C during July and August and then reduced by 4°C every two weeks beginning mid-September. In 1995, temperatures were set to fluctuate from 10°C (from 24:00 to 08:00 hr) to a high of 20°C at 15:00 hr, at increments of 1–2°C/hr, to simulate temperatures we had recorded with soil probes and a data logger at the Queensbury seed orchard during July and August in previous years. The high and low temperatures were reduced by 2°C every three to four weeks from September 1 to October 23, 1995. Pupae from Skimikin were stored at 20–22°C from July to mid-November and then at 4°C until early December. Pupae from all three locations in both years were overwintered in a cold room in constant darkness and mean temperature of 2°C (1–3°C) until mid-April, when they were returned to an environment chamber set at 11L:13D, 70% relative humidity, and a temperature of 9–10°C. The temperature was increased to 15–16°C over a two-week period and held constant until emergence was complete.

Upon emergence, males and females were placed in 37- × 28- × 29-cm screen cages in a controlled environment chamber with temperatures fluctuating between 9 and 21°C, 70% relative humidity, and a 14L:10D photoperiod. In 1995 and 1996, flies were provided with seed cones on host foliage, water, and the agar–honey–yeast diet described earlier. In 1996, flies were also provided with skim milk powder, buttermilk powder, and white spruce pollen cones as additional potential food sources to promote sexual development and mating. Examinations of eggs laid during experiments after two weeks of rearing at 20°C showed that all females collected in the field in 1995 were mated but that almost all laboratory-reared females (used in Fredericton in 1996 and in Skimikin during both years) were not.

Individual females were placed in a small, plastic aerated cage (12.5 × 7 × 7 cm) containing two previously caged cones on the same shoot in water. Only adjacent cones of similar size and shape were selected. After a female laid an egg and walked around while touching her mouthparts on the cone (hereafter referred to as marking), she was removed and a second female placed in the cage. The location of the first egg laid by the second female was recorded.

Replicates were only included in analyses if the second female contacted both cones before oviposition and engaged in marking behavior following oviposition. In all experiments, all cones were dissected to verify the presence/absence of eggs. If females avoid occupied cones, the second female should lay on the cone without an egg. If females do not avoid occupied cones, oviposition should be random and the second female should lay 50% of her eggs on each cone.

To determine whether avoidance of cones with eggs was due to the marking behavior observed following oviposition, we repeated this experiment, but following oviposition, we hit the cage to dislodge the fly from the cone before marking occurred. A second female was then introduced into the cage and we recorded whether she laid her first egg on the cone with or without an egg. If an oviposition-deterring substance is deposited while marking, and if females cannot distinguish adhesive or eggs, then eggs of the second female should be laid randomly on the two cones. The two experiments above were repeated in 1996 with a second (i.e., different) as well as the same female to make the choice between cones with and without eggs.

The influence of eggs (and of any associated adhesive substance that may or may not be present) on female oviposition site selection was also investigated in field bioassays at Skimikin in 1995. At the beginning of egg-lay, we dissected cones at Skimikin and removed cone scales bearing an egg. Using a paired design, on six trees we pinned either: (1) a scale containing an egg or (2) a non-egg-bearing scale from an unattacked cone on previously caged cones not containing eggs. The two cones in each cone pair were of similar size and appearance. Treatments were assigned randomly to cones within a pair by flipping a coin. Twenty-five pairs of cones (i.e., 25 cones per treatment) were treated on May 20 and 48 cone pairs on May 21. All cones were collected on May 23 after two and three days exposure, respectively, to the wild fly population.

Morphological Location of Active Compound(s). Field and laboratory bioassays were carried out to identify which body region contained one or more compounds that inhibited oviposition. In Skimikin in 1995, we placed females for 8 min in a freezer at $-5°C$ to reduce movement and then quickly separated the head and thorax from the abdomen using a scalpel. Water was added to ground body parts (ca. 0.05 ml of water for every female) to make solutions of the head + thorax or of the abdomen. Sixteen sets of three previously caged cones were selected on three trees on 19 May, and 51 sets of three cones were selected on ten trees on 20 May. Fresh body part extracts were made just prior to application each day. The three adjacent cones selected for each replicate were similar in size and appearance. One of the three treatments was assigned to each cone by drawing straws of different lengths. One female equivalent of head + thorax solution was applied to one cone in each set; one female equivalent of abdomen solution was similarly applied to another cone; and water was

applied to the remaining cone to serve as a control. Cones were identified with colored pins. Cones were removed after 48 hr, returned to the laboratory, dissected under a binocular microscope, and the number of eggs recorded.

Laboratory bioassays were carried out in Fredericton in 1996, with water solutions of female and male body parts obtained as described above. Individual females were placed in large screen cages in a controlled environment chamber set at 70% relative humidity, temperature fluctuating from 9 to 21°C (low at 02:00, high at 14:00 hr) and a 15L:9D regime. Each female was provided diet, water, and a white spruce shoot bearing four seed cones at a developmental stage receptive to egg-lay. The end of the shoot was freshly cut and immersed in water. Each cone received one of four treatments, consisting of one female or male equivalent of a body extract or a water control. The order of treatments on cones was systematically placed so that each treatment appeared on the most apical cone on the twig roughly the same number of times to avoid bias among treatments due to any subtle differences in cone quality. Flies were allowed to oviposit for 24 hr. Cones were then removed and dissected to record the number of eggs per treatment. There were three bioassays. In the first, we compared oviposition by 34 females on cones treated with either: (1) female abdomens, (2) female heads and thoraxes, (3) males abdomens, or (4) water. In the second, we compared oviposition by 17 females on cones treated with either: (1) female abdomens, (2) female heads and thoraxes, (3) male heads and thoraxes, or (4) water. In the third, we compared oviposition by 20 females on cones treated with either: (1) female abdomens, (2) female heads, (3) female thoraxes, or (4) water.

Analysis. G tests were used to test the hypothesis that eggs were laid randomly in laboratory and field bioassays (i.e., that egg-lay should be 50:50 in bioassays with cone pairs; 33.3:33.3:33.3 in bioassays with three treatments, etc.) (Zar, 1996). Yates's correction for continuity was applied to G tests with only one degree of freedom (Sokol and Rohlf, 1981). Comparisons for which the null hypothesis (Ho) was not rejected were subjected to retrospective power analysis for chi-square with PASS 6.0 (Hintze, 1996) to determine the probability of rejecting a false null hypothesis with the given sample size and a significance level (α) of 0.05. We considered a twofold difference in the proportion of eggs laid between treatments to be biologically significant and calculated the effect size accordingly.

RESULTS

Behaviors Associated with Oviposition. After alighting on cones, females walked around the cone, occasionally touching their mouthparts to the cone surface, and probed scales with their ovipositor. Females oviposited between cone scales and then walked around the cone while touching their mouthparts

to the cone (i.e., marking). At Skimikin in 1995, females in laboratory bioassays spent 77.0 ± 14.5 sec ovipositing ($\bar{X}$ ± SD) and 30.0 ± 17.3 sec marking (N = 3) following oviposition.

There was a large population of cone flies at Queensbury in 1995, which allowed us to document fly behavior both in the field and in laboratory bioassays carried out with females collected in the field. Females marked cones following 17 of 21 apparent oviposition bouts that we observed in the field. Dissections of 13 of the 17 marked and of all four unmarked cones showed that females had oviposited in all marked cones but in only two of four unmarked cones. In the field, females oviposited 62.3 ± 68.3 sec (N = 13) after alighting on cones. The duration of oviposition was 68.7 ± 22.5 sec (N = 15) and 101.1 ± 49.0 (N = 23), respectively, in the field and laboratory. The duration of marking following oviposition was 80 ± 50.7 sec (N = 13) and 84 ± 61.1 sec (N = 23), respectively, in the field and the laboratory.

Influence of Eggs and Postoviposition Behavior. Most flies encountering one cone on which a female had oviposited and marked and another unoccupied cone laid on the unoccupied cone (Table 1). Results for laboratory bioassays in Skimikin in 1995 on a small sample size were not significant (the probability of rejecting the null hypothesis, or power, was only about 10%), but they were significant for bioassays carried out in Fredericton and when replicates in both locations were pooled (Table 1). Results were similar in 1996. When individual females were given a choice between an unoccupied cone and a cone on which

TABLE 1. EGGS LAID BY *Strobilomyia neanthracina* PRESENTED WITH UNATTACKED CONE AND ONE OVIPOSITED ON AND MARKED BY THE SAME OR A DIFFERENT FLY IN LABORATORY EXPERIMENTS, WITH INTERIOR SPRUCE CONES AT SKIMIKIN, AND WHITE SPRUCE CONES AT FREDERICTON, 1995 AND 1996

Location	Year	2nd oviposition by	Cones with: Egg and marking	Cones with: No egg or marking	G statistic[a]	Power
Skimikin	1995	Different female	3	6	0.448	0.10
Fredericton	1995	Different female	8	31	13.170***	0.94
Combined	1995	Different female	11	37	13.685***	0.95
Skimikin	1996	Same female	7	18	4.114**	0.52
Skimikin and Fredericton combined	1996	Different female	7	17	3.459*	0.45

[a]G statistics adjusted by Yates correction. *, **, *** Egg lay differs from a 50:50 (random) ratio ($0.10 > P > 0.05$, $P < 0.05$ and $P < 0.001$, respectively).

they or a different female had previously oviposited and marked, they oviposited preferentially on the unoccupied cone (Table 1). However, females laid eggs randomly when presented with an unoccupied cone and one on which another female had previously oviposited but not marked in both 1995 (10 vs. 13 eggs, respectively; $G = 0.174$, ($P > 0.50$, power = 0.24) and 1996 (7 vs. 12 eggs, respectively; $G = 0.857$, $P > 0.25$, power = 0.36). Similarly, egg-lay was random when a female was presented with an unoccupied cone and one on which she had previously oviposited but not marked (10 vs. 15 eggs, respectively; $G = 0.646$, $P > 0.25$, power = 0.28).

In field bioassays at Skimikin, cones with a pinned, egg-bearing scale received a total of 31 eggs compared to 44 eggs in cones with a pinned, non-egg-bearing scale, but the difference was not significant ($G = 1.93$, $P > 0.10$, power = 0.79).

Morphological Location of Active Compound(s). In a field study at Skimikin in 1995, cone flies laid fewer eggs on cones covered with water extracts of female abdomens or water extracts of female heads and thoraxes than on cones that had received only water (Table 2).

In the first laboratory bioassay testing the influence of body extracts on oviposition site selection at Fredericton in 1996, 18 of 37 female flies laid a total of 67 eggs. Thus, the expected number of eggs per treatment under the null hypothesis would be 67/4 or 16.75 eggs per cone. The actual egg distribution differed significantly from the expected with the fewest eggs laid in cones painted with extracts of female heads and thoraxes (Table 3). In the second bioassay, seven of 17 females laid a total of 35 eggs. The distribution of eggs differed significantly among the treatments, with fewer eggs in the cones painted

TABLE 2. EGGS LAID BY *Strobilomyia neanthracina* IN INTERIOR SPRUCE CONES COVERED WITH WATER EXTRACTS OF FEMALE ABDOMENS, FEMALE THORAXES + HEADS, OR WATER ONLY (CONTROL) DURING TWO 48-HR PERIODS AT SKIMIKIN, 1995

		Extract				
Date[a]	N[b]	Control	Head + thorax	Abdomen	G statistic[c]	Power
May 19–21	47	26	12	16	5.621*	0.74
May 20–22	51	56	27	21	19.143***	0.95
Combined	98	82	39	37	23.029***	0.99

[a] Date when cones were exposed to ovipositing cone flies.
[b] N, number of cones per treatment.
[c] G statistics adjusted by Yates correction. *, *** Egg-lay differs from 33.33 : 33.33 : 33.33 (random) ratio ($0.10 > P > 0.05$ and $P < 0.001$, respectively).

TABLE 3. EGGS LAID BY FEMALE *S. neanthracina* IN WHITE SPRUCE CONES PAINTED WITH WATER (CONTROL) OR WATER-BASED EXTRACTS OF CONE FLY BODY PARTS IN THREE SEPARATE LABORATORY BIOASSAYS CONDUCTED AT FREDERICTON, 1996

Treatment	Bioassay 1[a]	Bioassay 2[b]	Bioassay 3[c]
Female abdomen	19	4	9
Female head & thorax	6	6	
Female head			4
Female thorax			1
Male abdomen	17		
Male head and thorax		1	
Water (control)	25	24	7
Total eggs laid	67	35	21
Expected egg lay if random	(16.75)	(8.75)	(5.25)

[a] $G = 12.99$, $P < 0.005$ for Ho that egg-lay is equal among the four treatments. $G = 1.69$, $P > .25$, power = 0.75, for Ho that egg-lay is equal among female abdomen, male abdomen, and control. $G = 10.49$, $P < 0.005$ for Ho that egg-lay is 3:1 between the combined treatments of female abdomen + male abdomen + control vs. female head and thorax (adjusted by Yates correction for continuity), i.e., significantly fewer eggs than expected on female head and thorax.

[b] $G = 33.3$, $P < 0.001$ for Ho that egg-lay is equal among the four treatments. $G = 3.99$, $P > 0.10$, power = 0.34, for Ho that egg-lay is equal among female abdomen, female head and thorax, and male head and thorax, i.e., no difference among fly extracts but each have fewer eggs than the control.

[c] $G = 8.24$, $P < 0.05$ for Ho that egg-lay is equal among the four treatments. $G = 1.99$, $P > 0.25$, power = 0.35 for Ho that egg-lay is equal among female abdomen, female head and control. $G = 5.19$, $P < 0.025$ for Ho that egg-lay is 3:1 between the combined treatments of female abdomen + female head + control vs. female thorax (adjusted by Yates correction for continuity), i.e., significantly fewer eggs than expected on cones with female thorax extract.

with any of the fly extracts compared with water controls. There were no significant differences in egg lay between the fly extracts, but the power of the test was relatively low (0.34) (Table 3). In the third bioassay, six of 20 females laid a total of 21 eggs. Cones with extracts of female thorax contained significantly fewer eggs than those painted with female head, female abdomen, or water controls (Table 3).

DISCUSSION

In general, females avoided laying on cones on which they or other females had oviposited and marked. The avoidance of marked cones with eggs was attributable to marking and not to the presence of eggs because females did not

avoid unmarked cones that contained an egg. Our laboratory and field bioassays clearly show that *S. neanthracina* females do not distinguish eggs, which are often concealed under scales, or any adhesive substance that may be used to attach eggs to scales, as was the case for the only other anthomyiid fly reported to deposit a HMP (Zimmerman, 1979).

Strobilomyia neanthracina spent a considerable period of time walking over cones before ovipositing. This behavior greatly increases the chances that a female will encounter the HMP if it is present. Some flies left a marked cone after only contacting it with their feet, suggesting that the HMP can be perceived by contact tarsal chemoreceptors. Females occasionally touched their mouthparts to cones while walking on them and also often inserted their ovipositor in between several cone scales before ovipositing, and thus HMP receptors may also be associated with the mouth and/or ovipositor. HMPs are usually perceived by contact chemoreceptors on tarsi and the abdomen (Crnjar et al., 1978; Klijnstra and Roessingh, 1986; Städler et al., 1994) although Kouloussis and Katsoyannos (1991) postulated that the almond seed wasp was also able to perceive volatiles of its HMP with antennal receptors.

A small proportion of females did not mark following oviposition. It is possible that the propensity to mark cones is affected by various factors not investigated in the present study, such as female age (Averill and Prokopy, 1987b) or egg load (van Randen and Roitberg, 1996).

Our study indicates that preoviposition, oviposition, and postoviposition behaviors of mated *S. neanthracina* females are similar in the laboratory and field. Although they found that the incidence of marking may differ in the field and in a greenhouse, in a greenhouse study, Brockerhoff and Turgeon (personal communication) measured durations of oviposition and marking by *S. neanthracina* that were similar to those that we report here. Because females used at Fredericton in 1995 were mated, whereas most females used in Fredericton in 1996 and in Skimikin in both years were not, our study also indicates that preoviposition, oviposition, and postoviposition behavior of mated and unmated cone flies is qualitatively similar. Unmated females tended to mark cones for a shorter period of time but they still marked hosts after oviposition and avoided cones marked by unmated females. Similarly, body extracts of unmated females deterred oviposition. These results support those of Quiring and McNeil (1984b), who reported that body extracts containing HMP of unmated females of a leaf mining fly reduced oviposition as long as females had access to food for ≥ 15 hr.

Previous studies with cone flies exploiting larch (McClure, 1995) and spruce (Fidgen, unpublished data; Sweeney, unpublished data) have reported that multiple eggs are laid on cones in years when seed cones are scarce. This could result from a rapid decrease in the efficacy of the HMP with time and/or because females oviposit on marked hosts when unmarked hosts are not readily available.

Although HMPs can remain effective for considerable periods of time under dry laboratory conditions (Katsoyannos, 1975; Prokopy, 1975; Prokopy et al., 1978; Schoonhoven et al., 1981; Averill and Prokopy, 1987b; Pittara and Katsoyannos, 1990), their efficacy can be much shorter lived in nature (Quiring and McNeil, 1984c; Klijnstra and Schoonhoven, 1987), especially if exposed to heavy rain (Averill and Prokopy, 1987b). Similarly, although frequent encounters with HMP can promote dispersal (Roitberg et al., 1982, 1984; Quiring and McNeil, 1987), females will lay on hosts with HMP when deprived of unmarked hosts for a period of time (Roitberg and Prokopy, 1983).

Utilization of mouthparts by adult *S. neanthracina* to deposit HMP differs from the mechanism reported for adults of other species, which use their ovipositor to deposit HMP following oviposition (Zimmerman, 1979; Prokopy et al., 1984; Roitberg and Prokopy, 1987). Female head extracts deterred oviposition in three of four studies, and thorax extracts deterred oviposition in four of four studies, further supporting the hypothesis that a marking pheromone is contained in these body regions and not in the abdomen. Female abdominal extracts only deterred oviposition in two of four experiments. Male abdomens did not contain any chemicals that deterred oviposition, but male thoraxes may contain some as yet unknown oviposition deterrent.

These experiments support the hypothesis that, following oviposition, *S. neanthracina* females mark cones with a pheromone that deters oviposition by females subsequently encountering these cones. Our study appears to explain the uniform egg distribution observed in nature for *S. neanthracina* (Sweeney, unpublished data) and suggests that HMP may also be responsible for the uniform egg distributions observed for *S. laricis* and *S. viaria* (McClure, 1995) and for *S. appalachensis* (Sweeney and Turgeon, 1994).

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INHIBITION OF METHANE CONSUMPTION IN FOREST SOILS BY MONOTERPENES

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Abstract—Selected monoterpenes were tested for their ability to inhibit atmospheric methane consumption by three forest soils from different vegetation types and by the cultured methanotrophic strain, *Methylosinus trichosporium* OB3b. Subsurface soil from coniferous (*Pinus banksiana*), deciduous (*Populus tremuloides*), and mixed hardwood (*Tsuga canadensis* and *Prunus pensylvanica*) stands was used under field-moist (bulk and intact cores) and slurry conditions. Most of the hydrocarbon monoterpenes tested significantly inhibited (40–100%) methane consumption by soils at environmentally relevant levels, with (−)-α-pinene being the most effective. With the exception of β-myrcene, monoterpenes also strongly inhibited methane oxidation by *Methylosinus trichosporium* OB3b. Carbon dioxide production was stimulated in all of the soils by the monoterpenes tested. In one case, methane production was stimulated by (−)-α-pinene in an intact, aerobic core. Oxide and alcohol monoterpenoids stimulated methane production. Thus, monoterpenes appear to be potentially important regulators of methane consumption and carbon metabolism in forest soils.

Key Words—Methane consumption, inhibition, monoterpenes, forest soils, methanotrophs, incubations, soil cores, pinene.

INTRODUCTION

Monoterpenes (C_{10} unsaturated hydrocarbons) are volatile organic compounds of biogenic origin and are major components of plant (essential) oils. They are particularly abundant in families such as the Pinaceae, but their distribution is widespread (Dev, 1982; Lerdau et al., 1997). Monoterpenes are allelopathic

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compounds and are part of the plant's defense against insects and fungal pathogens (Dev, 1982; Lerdau et al., 1997). Their antimicrobial activity against certain bacteria is also well known (Subba et al., 1967). They have a variety of commercial and industrial applications (Subba et al., 1967; Dev, 1982; Chang and Oriel, 1994; Misra et al., 1996) as well as an environmentally important role. Nonmethane hydrocarbon emissions from natural sources to the atmosphere consist in large part of monoterpenes (Mooney et al., 1987; Guenther et al., 1994; Lerdau et al., 1997), which are in turn involved in several atmospheric reactions (White, 1994; Lerdau et al., 1997).

It has been suggested that monoterpenes influence the N cycle in forest soils by inhibiting autotrophic nitrification (White, 1986, 1994) and that this inhibition is a result of direct action of the compounds on ammonia monooxygenase (AMO) (White, 1988, 1990), the first enzyme in the process. This led to speculation that methane oxidation, a process that depends on a methane monooxygenase (MMO) enzyme with function and properties similar to those of AMO (Bédard and Knowles, 1989), might also be affected by monoterpenes in forest soils (White, 1994). In this study, the effect of monoterpene additions to different forest soils as slurries and under more field-relevant conditions was tested. A variety of hydrocarbon monoterpenes, as well as some oxide and alcohol forms, were used to determine differences in inhibition between compounds. Compounds that are common in forest environments were chosen.

METHODS AND MATERIALS

Sites and Sampling. Forest soil samples were obtained from stands of aspen (*Populus tremuloides*) and young jackpine (*Pinus banksiana*) at the BOREAS northern study site near Thompson, Manitoba, Canada. The soils, designated Aspen and Pine, respectively, were described previously (Amaral and Knowles, 1997a,b) and correspond to Pine 1 and Aspen 1 soils of Savage et al. (1997). Soil was also taken from a mixed hardwood–conifer forest in Durham, New Hampshire, USA, with dominant vegetation of eastern hemlock (*Tsuga canadensis*) and pin cherry (*Prunus pensylvanica*) (Crill, 1991). Properties of this soil, designated as Mixed, are given by Crill (1991) and Amaral and Knowles (1997a). Briefly, all soils used had an upper organic layer, 3–6 cm thick, underlain by a mineral layer of clay or sand. Aspen and Pine soils were collected using polyvinylchloride core tubes (10 cm diameter and 20–30 cm long) as previously described (Amaral and Knowles, 1997b). Some soil cores were later extruded and sectioned to determine where in the soil column methane consumption occurred (Amaral and Knowles, 1997b). Samples of the Mixed soil were obtained in bulk from soil column depths of 0–3 cm and 7–12 cm and

stored in plastic bags. Soils were collected in the spring and summer of 1996 and stored at 4°C until use.

Incubation Experiments. The effect of monoterpenes on methane consumption was tested on various depth horizons of the Pine, Aspen (5–10 cm), and Mixed (0–3 cm and 7–12 cm) soils, using field-moist samples and slurries. In one experiment, field-moist Mixed soil fractions were tested both prior to and after preincubation (two to three weeks) with high levels of methane (5%). Stones and roots were removed with a coarse sieve (4-mm mesh) and 5–10 g of field-moist soil were incubated in 125-ml Erlenmeyer flasks, capped with Suba-Seal serum stoppers (W.H. Freeman Co., Barnsley, UK). Incubations were carried out at 25°C and under room atmosphere (ambient methane 1.8–2.0 ppm) in the presence and absence of monoterpene vapors. Monoterpene vapors were introduced by placing a test tube containing a strip of monterpene-soaked (50–100 μl) filter paper in selected flasks. Alternatively, 10 ml of soil slurry (1:5 soil weight to water volume) were incubated in 58-ml serum bottles on a rotary shaker (250 rpm) under the conditions described above. Pure monoterpenes (>98%) were added directly to each flask, using a Hamilton glass microsyringe, to final concentrations of 1–10 mg/g soil. Monoterpenes used in this study were (−)-α-pinene, α-terpinene, and β-myrcene from ICN Biochemicals, Aurora, Ohio; and (+)-α-pinene, (−)-β-pinene, (*R*)-(+)-limonene, α-pinene oxide, α-terpineol, and (±)-linalool from the Aldrich Chemical Co., Milwaukee, Wisconsin. Before addition to flasks, monoterpenes were degassed under vacuum and stored under an atmosphere of pure helium to minimize carryover of significant levels of methane.

Methane and carbon dioxide in the headspaces of the incubation vessels were determined by gas chromatography using flame ionization and thermal conductivity detection, as previously described (Roy et al., 1994; Amaral and Knowles, 1997a,b). Headspace gas samples of 0.5–1.0 ml were removed by syringe for analysis four times over an incubation period of 12–15 hr.

Other incubations were done with intact Aspen soil cores. Five core tubes were sealed (air-tight) at both ends with plastic caps and Terostat compound (Teroson GmbH, Heidelberg, Germany). The top cap was predrilled and fitted with a rubber stopper and Suba-Seal assembly for sampling headspace gases. The gas-sampling assembly could be removed to equilibrate the core headspace with the atmosphere. Cores were first incubated (12 hr) without monoterpene addition. After reequilibration of the headspace with the atmosphere (2 hr), a 2-cm square of filter paper was placed in each core and 100 μl (−)-α-pinene added to the paper. The core tubes were again stoppered and methane consumption determimed as described above. The process was repeated to determine the effect of another (−)-α-pinene addition (500 μl) on methane consumption by the cores. The total pore and headspace of each core was

determined as described in Dunfield et al. (1995). Headspace volumes of cores varied from about 500 to 900 ml.

Atmospheric methane consumption, a first-order process, was calculated from rate constants derived from log-transformed time-course data. Where methane production occurred, rates were calculated from the slopes of regressions ($r^2 > 0.98$) from the initial, linear portion of the production curve. Most incubations were carried out in duplicate or triplicate and values are reported as the means of the incubations $\pm$ SEM. Errors reported for determinations on single samples, such as individual cores, are the standard errors (SE) of the slopes of regressions obtained using the SigmaPlot graphics program (Jandel Scientific Software).

Soil moisture content was determined gravimetrically after drying aliquots at 110°C overnight. These values were used to calculate activities reported on a per gram dry weight basis.

Pure cultures of the methanotroph *Methylosinus trichosporium* OB3b (a gift of R. S. Hanson, University of Minnesota) were grown in a nitrate mineral salts (NMS) medium (Whittenbury et al., 1970) under an atmosphere of 20% methane and 80% air. Erlenmeyer flasks (125 ml) with 20 ml of NMS and 1.5 mg (approx. 1.1 mM final concentration) of selected monoterpenes were inoculated with a mid-log phase culture of *M. trichosporium* OB3b to give a final OD_{600} of 0.04. The flasks were incubated at 25°C on a rotary shaker (230 rpm) under an atmosphere of 20% methane and 80% air. The amount of methane consumed after three days was determined by gas chromatography, as described above.

RESULTS

Addition of monoterpenes inhibited atmospheric methane consumption in the three soils tested. Pine, Aspen and Mixed, under both field-moist and slurry conditions (Tables 1 and 2, Figure 1). Inhibition was concentration dependent, with 10 mg, but not 1 mg, monoterpene per gram dry weight of soil significantly inhibiting activity in Pine soil slurries (Table 1). Despite the equal loading and similar solubilities of these compounds (Weidenhamer et al., 1993), (−)-α-pinene showed greater inhibitory potential (96%) than the other monoterpenes used with this soil. At half the loading rate (5 mg/g dry weight soil), which approaches natural levels of monoterpenes in forest floor litter (Wilt et al., 1993), Aspen soil slurries were also inhibited (Table 2). However, the pattern of inhibition differed from that in the Pine soil. For example, in Aspen soil, (−)-α-pinene gave inhibition more or less equal to that of α-terpinene and (*R*)-(+)-limonene. β-Myrcene also showed an inhibition potential relatively lower in Aspen soil (only 15%) than in Pine soil (62%), while some of the other monoterpenes used gave similar inhibition (61–66%) in both Pine and Aspen soil slurries, even at lower concentrations.

TABLE 1. EFFECT OF SELECTED MONOTERPENES ON ATMOSPHERIC METHANE CONSUMPTION BY SLURRIES OF PINE FOREST SOIL (5–10 cm DEPTH)[a]

Supplement (mg/g dry soil)	CH_4 consumption (nmol/g/day ± SEM)	Inhibition (%)
None	4.59 ± 0.32	
(−)-α-Pinene		
1	4.54 ± 0.05	0
10	0.18 ± 0.18	96
α-Terpinene		
1	4.91 ± 0.23	0
10	1.38 ± 0.72	70
β-Myrcene		
1	4.38 ± 0.16	0
10	1.75 ± 0.28	62
(*R*)-(+)-Limonene		
1	4.63 ± 0.28	0
10	1.78 ± 1.38	61

[a]Soil slurries were incubated and shaken for 15 hr. Data are means of duplicate incubations.

TABLE 2. EFFECT OF SELECTED MONOTERPENES ON ATMOSPHERIC METHANE CONSUMPTION BY SLURRIES OF ASPEN FOREST SOIL (5–10 cm DEPTH)[a]

Supplement	CH_4 consumption (nmol/g/day ± SEM)	Inhibition (%)
None	0.42 ± 0.04	
(−)-α-Pinene	0.17 ± 0.02	59
(+)-α-Pinene	0.22 ± 0.01	46
(−)-β-Pinene	0.24 ± 0.01	42
α-Terpinene	0.14 ± 0.01	66
β-Myrcene	0.35 ± 0.01	15
(*R*)-(+)-Limonene	0.15 ± 0.01	65

[a]Soil slurries were incubated and shaken for 15 hr. Monoterpenes were added at a rate of 5 mg/g dry soil. Data are means of duplicate incubations.

Addition of oxide and alcohol forms of monoterpenes to Mixed soil (7–12 cm) slurries resulted in methane production (up to 4.5 nmol/g/day), whereas addition of hydrocarbon forms resulted in inhibition of methane consumption during the incubation period (Table 3). Oxygen was still abundant (≈15%) in the headspace of both methane-producing and methane-consuming samples at

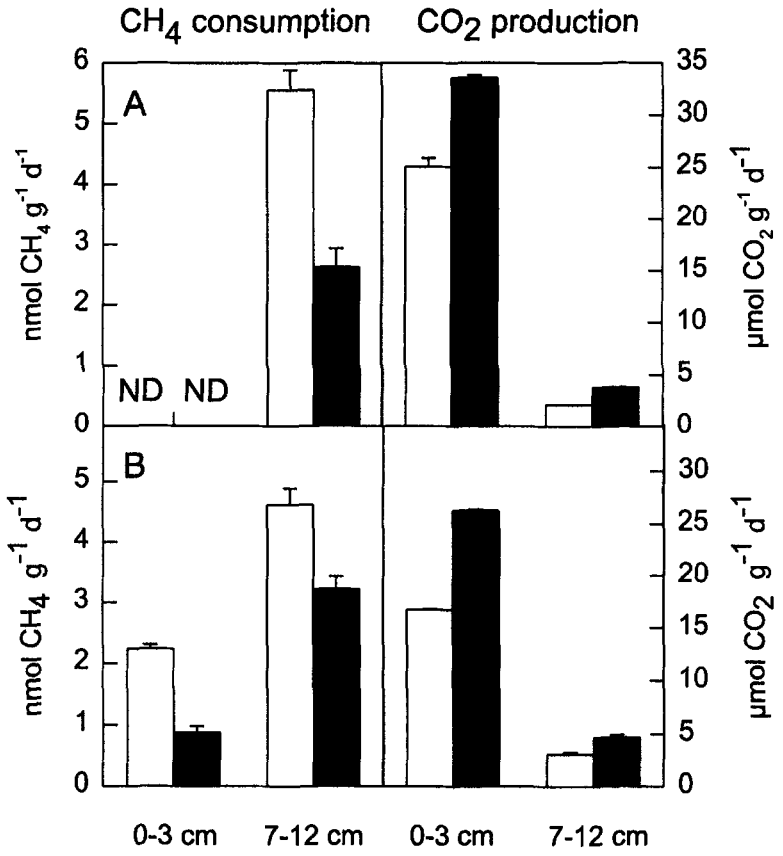

FIG. 1. Atmospheric methane consumption and carbon dioxide production by Mixed forest soil collected from 0–3 and 7–12 cm depths in the absence (open bars) and presence (solid bars) of (−)-α-pinene vapours, before (A) and after (B) methane enrichment of the soils. Incubations were done with field-moist soils (70.8% and 28.3% water content, respectively). Enriched soils were preincubated with 5% methane (balance was room air) for two to three weeks until activity was observed in the 0- to 3-cm sample. Data are means of duplicate incubations (±SEM). ND: not detected.

the end of the incubation (data not shown). Methane production in aerobic forest soil was previously observed when methanotrophy was specifically inhibited by methyl fluoride (Yavitt et al., 1995). (−)-α-Pinene was strongly inhibitory, as observed for the other soils. The β isomer of pinene was less inhibitory than the α isomer (Table 3), as was seen in Aspen soil (Table 2).

Field-moist Mixed soil, from 0–3 and 7–12 cm depths (70.8% and 28.3%

TABLE 3. EFFECT OF SELECTED MONOTERPENES ON ATMOSPHERIC METHANE OXIDATION BY SLURRIES OF MIXED SOIL (7–12 cm DEPTH)[a]

Supplement	CH_4 consumption (nmol/g/day ± SEM)	Inhibition (%)
None	1.90 ± 0.06	
(−)-α-Pinene	ND	100
α-Terpinene	ND	100
(−)-β-Pinene	0.48 ± 0.02	75
β-Myrcene	0.31 ± 0.05	84
α-Pinene oxide	−4.47 ± 0.27	−235
α-Terpineol	−2.59 ± 0.02	−136
(±)-Linalool	−0.31 ± 0.01	−16

[a]Soil slurries were incubated and shaken for 25 hr. Monoterpenoids were added at a rate of 10 mg/g dry soil. Data are means of duplicate incubations. ND = not detected.

water, respectively), was exposed to (−)-α-pinene vapour (Figure 1). Both preexisting and induced methane consumption activities were inhibited by up to 50%, demonstrating the ability of gaseous monoterpenes to reach sites of methane consumption in moist soils despite their low water solubility (Weidenhamer et al., 1993). In contrast, production of carbon dioxide was stimulated by up to 58% with α-pinene addition, indicating that the observed decrease in methane consumption was not due to a general negative effect of pinene on the soil microflora. Similar increases in carbon dioxide production occurred with the other soils and monoterpenes used (data not shown).

Core incubations were used to test the effect of monoterpenes on soil methane consumption under conditions approaching those in the environment. Addition of a (−)-α-pinene-saturated filter paper square to the surface of the soil simulated the high monoterpene levels expected in the surficial layers of forest soils (White, 1994). Four of five Aspen cores showed significant decreases in methane consumption upon a first addition of 100 μl (≈ 86 mg) of (−)-α-pinene (allowed to volatilize from filter paper) (Figure 2). Venting the cores to allow pinene vapors to escape and to equilibrate the core headspace with the atmosphere (6 hr) did not affect the depressed activity levels. A further, greater, addition of (−)-α-pinene did not affect the activity levels either, suggesting that some sites for methane consumption in the soil were not accessible to the monoterpene. Methane consumption remained depressed even after two days of venting the cores to the atmosphere, with only a slight recovery seen in cores 2 and 4 (Figure 2). In one core (5), (−)-α-pinene greatly stimulated preexisting net methane production and flux (Figure 2). This observed stimulation suggests

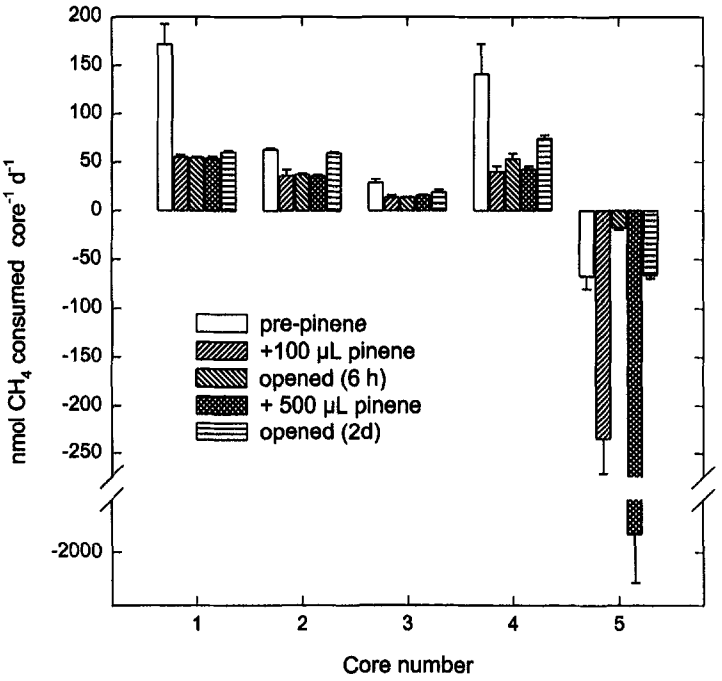

FIG. 2. Effect of two successive (−)-α-pinene additions to five individual intact cores of Aspen soil. Values reported (±SE) are from measurements made over a period of 12 hr after each monoterpene addition. The core tubes were left capped for a further period of 36 hr, to prevent loss of (−)-α-pinene. Cores were then opened to the atmosphere for the times shown in the legend before initiating the next measurement.

that, in the presence of monoterpene, any measure of methane consumption is potentially only a net rate.

Cultures of the methane-consuming bacterium *M. trichosporium* OB3b were also inhibited by monoterpene additions at the levels added to the above soil incubations (Figure 3). Under these experimental conditions, almost complete inhibition of methane oxidation occurred with (−)-α-pinene, (*R*)-(+)-limonene, and α-terpinene, also strong inhibitors in soils (see above). However, unlike the situation in the soil incubations, β-myrcene had no effect on methane oxidation by the bacterium.

DISCUSSION

The widespread occurrence of monoterpenes in higher plants (Dev, 1982; Lerdau et al., 1997) and the known antimicrobial activity of these compounds

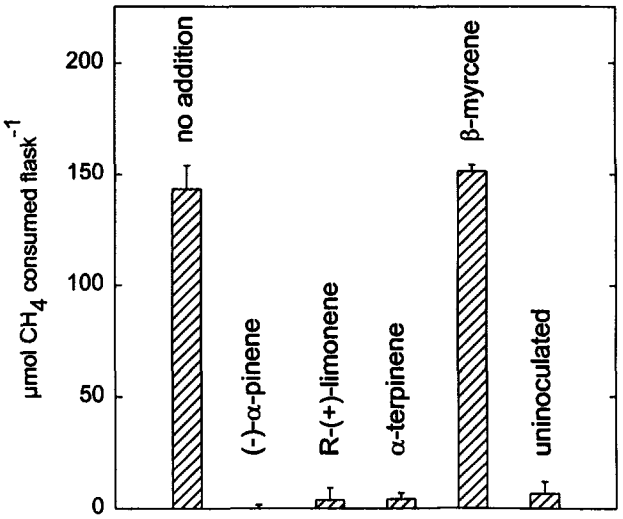

FIG. 3. Effect of various monoterpenes on methane oxidation by *Methylosinus trichosporium* OB3b. Cultures (10 ml; OD_{600} = 0.04) were incubated with 1.5 mg of monoterpene and methane oxidation measured after three days of exposure to monoterpene. The results are means of triplicate incubations (±SEM).

point to their potential role in controlling soil microbial characteristics. In fact, the impact of monoterpenes on soil microbes is likely to be complex since they stimulate activity and support growth of certain bacteria (Vokou et al., 1984; Vokou and Margaris, 1988; Misra et al., 1996) but inhibit others (Subba et al., 1967; Chang and Oriel, 1994). The results of our study suggest that bacteria responsible for atmospheric methane consumption in forest soils represent one microbial group that is negatively affected by these compounds.

Large amounts of monoterpenes are released by woodlands via foliar emission (Guenther et al., 1994), but processes such as litterfall and root exudation can deliver a significant quantity to forest soils. Little is known about the persistence of monoterpenes in forest soil. However, high levels, comparable to those in fresh foliage, are found in the upper litter layer, although they typically decrease exponentially with depth (White, 1994). Total monoterpene levels of 3.6 mg/g dry weight were reported for the senescent needle layer in a pinyon pine stand (Wilt et al., 1993). These levels are comparable to those used in the present study and lead to the conclusion that natural levels of monoterpenes in some forest soils might prevent methane oxidation in the litter layer. Typical depth profiles of methane consumption do indeed show no or low activity in the top of the soil column (Adamsen and King, 1993; Amaral and Knowles, 1997b). Although other factors are also involved (Amaral and Knowles, 1997a), soil monoterpene distribution may be important in controlling this activity profile.

Monoterpenes are also implicated in the inhibition of nitrification in forest soils (White, 1986, 1988, 1990, 1994) and may partly explain the low nitrification rates in the litter layers of many of these. It is postulated that these compounds have a direct effect on AMO (White, 1988), the enzyme initiating the nitrification pathway. Methane monooxygenase, the enzyme responsible for the oxidation of methane to methanol, shares may similarities with AMO, including a sensitivity to the same inhibitors (Bédard and Knowles, 1989). Experiments with pure cultures of the methanotrophs *M. trichosporium* OB3b (this study) and *Methylomonas* sp. (White, 1994) and the nitrifier *Nitrosomonas europaea* (Courtney et al., 1991) have shown these organisms to be sensitive to several monoterpenes, although by what mechanism is not yet clear. Besides inhibition of nitrification, the N cycle in forest soils may be impacted also by an increase in ammonium-N assimilation due to stimulation of carbon mineralization resulting from monoterpene additions (Bremner and McCarty, 1988; White, 1990).

Similarly, the C cycle of these soils is also impacted beyond the inhibition of methane oxidation. As well as observing an increase in carbon dioxide production, as previously reported (Vokou and Margaris, 1988), we also noted the potential for monoterpenes to enhance production of methane in forest soils. To our knowledge this is the first time such an effect has been reported for monoterpenes. This finding has some importance for the global methane budget. As suggested by White (1994), in regions such as the northern latitudes with monoterpene-rich coniferous vegetation and large areas of methane-producing environments, inhibition of methane oxidation may account for significantly greater methane fluxes to the atmosphere. Part of these fluxes may also be due to monoterpenoid stimulation of methane production.

In summary, we found that monoterpenes, added under ecologically relevant conditions, can greatly inhibit methane consumption in both forest soils and a methanotroph culture. The varied responses to structurally different compounds suggests that the extent of inhibition in nature will depend, among other factors, on the types of monoterpenes and their sources. In some cases, production of methane may be as significant an effect as the inhibition of methane consumption. In general, monoterpenes have the potential to be important regulators of carbon metabolism in some environments.

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GLYCINE PROTECTS AGAINST STRONG PROTEIN-DENATURING ACTIVITY OF OLEUROPEIN, A PHENOLIC COMPOUND IN PRIVET LEAVES

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Abstract—In previous studies, we reported the existence of a high concentration of free glycine in the digestive juice of several Lepidoptera larvae, particularly in the digestive juice of species that feed on the privet tree, *Ligustrum obtusifolium*. The water extract of privet leaves showed very strong protein-denaturing activity and lysine-decreasing activity, which closely resembled activity of oxidized polyphenolics. Addition of 1% glycine to the extract could completely inhibit these activities. Free glycine may be secreted into the digestive juice by larvae as an adaptive mechanism for chemical defense against its host plants. The protein-denaturing compound in privet leaves is present in the cytosol or in the vacuoles of the leaf cells. The compound does not show protein-denaturing activity without oxidation, but when mixed with intact organelles under low osmotic conditions to give an osmotic shock, a very high protein-denaturing activity is produced. Our results suggest that the privet tree is endowed with a defense mechanism in which a stable compound in the cytosol or in the vacuoles is activated into a chemically active denaturant by an enzyme present in the organelles (including chloroplasts) after the leaves are eaten by insects and the organelles are broken by osmotic shock or by digestive mechanisms. Based upon HPLC and NMR data, we conclude that the denaturing compound is oleuropein, an *o*-dihydroxyphenolic compound. This compound makes up 3% of the wet weight of privet leaves. The protein-denaturing activity of purified oleuropein activated by the leaf enzyme is high enough to account for all the denaturing activity in a water extract of privet leaves. The denaturing reaction is completely inhibited by free glycine. Our results suggest that the protein-denaturing activity and lysine-decreasing activity of privet leaves are caused by oxidized polyphenolics, and that some insects secrete free glycine to counter the denaturing activity of oxidized phenolics. The chemical mech-

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anism of counteraction by glycine is also considered. Free glycine in the midgut of insects probably protects proteins from denaturation by competing with proteins for oxidized phenolics.

Key Words—Glycine, digestive juice, Lepidoptera, privet tree, *Ligustrum obtusifolium*, plant-herbivore interactions, plant defense, protein denaturation, phenolics, oleuropein, lysine decrease, phenolic-protein binding, subcellular compartmentation, polyphenol oxidase, insect nutrition, *Brahmaea wallichii*.

INTRODUCTION

Phenolics are molecules occurring in a wide variety of plants in considerable quanities. They are known to exert many kinds of adverse effects on growth of herbivores. Plant phenolics (e.g., tannins) are considered to be defensive molecules of plants against herbivores and are assumed to play important roles in plant-herbivore interactions and coevolution (Feeny, 1968, 1970; Bernays, 1981; Rossiter et al., 1988; Appel, 1993). The adverse effects of phenolics on herbivores include digestion-inhibitory effects with denaturation and aggregation of digestive enzymes and proteins (Feeny, 1968, 1969; Appel, 1993) and reduction of protein quality (Felton et al., 1989, 1992a). Several studies have indicated that oxidized phenolics are responsible for these effects in many cases (Felton et al., 1989, 1992a; Felton and Duffey, 1991a; Hurrell et al., 1982; Pierpoint, 1969; Appel, 1993). These studies show that quinone formed by the oxidation of dihydroxyphenolics reacts covalently with the amino residue in the side chain of lysine in protein and that this covalent bond causes denaturation of protein and loss of lysine (Hurrell et al., 1982; Pierpoint, 1969; Felton et al., 1989, 1992a). Since lysine is an essential amino acid, the nutritive value of protein decreases (Felton et al., 1989, 1992a; Hurrell et al., 1982). In order to feed on plants that contain oxidized phenolics, herbivorous insects should have developed some adaptive mechanisms. Recent reports have revealed the existence of adaptive mechanisms in a number of insects, including the production of quinone reductase (Yu, 1987), ascorbate-reducing enzymes (Felton and Duffey, 1992), catalase (Felton and Duffey, 1991b), reducing conditions in the gut (Appel and Martin, 1990), and an acidic midgut (Felton et al., 1992b). Because the reactions of oxidized phenolics seem to start as soon as plants are eaten by herbivores, it is important for herbivores to develop protection mechanisms in the gut lumen and to make them work immediately after the feeding process to prevent dietary proteins from being attacked by oxidized phenolics. Therefore, existence of such immediate mechanisms in the midgut lumen was expected.

Recently, we detected a large amount of free glycine in the midgut contents and the digestive juices of several Lepidoptera larvae (Konno et al., 1996, 1997).

Evidence suggested that the free glycine was secreted into the gut lumen in the anterior part of the midgut by a glycine-specific transport mechanism (Konno et al., 1996). The amount of glycine differed markedly between species and seemed to depend on the host plant (Konno et al., 1997). Several species of Lepidoptera from different families that feed on the privet tree, *Ligustrum obtusifolium* (Oleaceae), exhibited large amounts of free glycine in the digestive juice, and the concentration reached 57 μmol/g (0.4%). A water extract of privet leaves showed very strong protein-denaturing activity, sufficient to make the protein completely nonnutritive. This denaturing property closely resembled the activity of oxidized phenolics in that the content of lysine decreased to 1/5 of that of the original protein (Konno et al., 1997). We also showed that the loss of lysine was the cause of the reduction of the nutritive value. An in vitro study indicated that the addition of 1% glycine to the leaf extract can completely inhibit this activity. These observations suggested that free glycine is secreted into the digestive juice by larvae as a mechanism to reduce the protein-denaturing activity of the privet leaf presumably caused by oxidized phenolics.

In this study, we examine the chemical basis of this phenomenon. First, we show that the protein-denaturing compound is accumulated in the cytosol or in the vacuoles of the leaf cells and that this compound is chemically stable. We demonstrate that this compound is activated by an activating enzyme(s) localized in organelles including chloroplasts. Second, we show through purification and identification by HPLC and NMR that the protein-denaturing compound of the privet leaf is oleuropein, an *o*-dihydroxyphenolic compound. Third, we show that glycine can inhibit the protein-denaturing activity of purified oleuropein. In this paper, we also present a chemical model for this phenomenon based on these data.

METHODS AND MATERIALS

Preparation of Water Extract of Privet Leaves. Prior to homogenization, leaves (4 g) were cut into small pieces with scissors. Then, the leaves were homogenized (30,000 rpm, 1 min) in five volumes of ice-cold distilled water (20 ml), by using a polytron-type homogenizer (Model NS-50, Physicotron) equipped with a generator shaft (10.5 mm diameter, model NS-10, Physicitron). The leaf pellet was removed by centrifugation (10,000*g*, 10 min), and the supernatant was collected as the water extract of privet leaves.

Preparation of Substrate Fraction (Denaturing Compound) Free of Activating Enzyme. To inactivate foliar enzymes, privet leaves were steamed (100°C, 4 min) prior to homogenization. The steamed leaves were homogenized with five volumes of ice-cold distilled water as described above. The leaf pellet was

removed by centrifugation (10,000*g*, 10 min), and the supernatant was collected as the substrate fraction.

Preparation of Enzyme Fraction (Organelle Fraction) Free of Substrate (Denaturing Compound). A modification of the method of Golbeck and Cammarata (1981) used for preparing intact chloroplasts and thylakoid-bound polyphenol oxidase was applied here to prepare a fraction that contains the activating enzyme but not the substrate. Organelles, including chloroplasts, were prepared by homogenizing 16 g of the privet leaves with 100 ml of ice cold 0.1 M sodium phosphate buffer (pH 7.0) containing 0.4 M sucrose. The homogenization was performed as described above. The homogenate was centrifuged at 1000*g*, 4°C for 2 min. The pellet was discarded and the supernatant containing organelles and the substrate was recentrifuged at 3000*g*, 4°C for 25 min. After recentrifugation, the supernatant containing the substrate was discarded. The pelleted organelles were resuspended and washed twice in 80 ml of the same buffer to remove the remaining substrate. After centrifugation at 3000*g* for 25 min, the pellet was resuspended in 20 ml of the same buffer, and this suspension of intact organelles was used as the enzyme fraction. To analyze the enzyme activity in organelles, it is necessary to give an osmotic shock to break organelles. However, under normal experimental procedures, the osmotic shock was automatically given when the organelle suspension was diluted into a reaction solution with low osmotic pressure.

Assays of Protein-Denaturing/Lysine-Decreasing Activity. All the assays were performed in 750 μl of 0.1 M sodium phosphate buffer (pH 7.0), containing 1% ovalbumin, 0 or 1% glycine, 500 μl of substrate (water extract of privet leaves, substrate fraction, purified fractions by HPLC, oleuropein solution, or catechol solution), and 0 or 100 μl of the enzyme fraction (organelle fraction). Assays were performed in triplicate at 25°C for 2 hr in 1.5 ml microfuge tubes, with shaking from time to time to allow the solution to come into contact with oxygen in the air. Five microliters of the reaction solution was then taken and applied to SDS-PAGE to determine the protein-denaturing activity. The degree of denaturation was determined by the band pattern (disappearance of the original ovalbumin band and emergence of a high molecular weight band of protein aggregation formed by the denaturing activity). For amino acid analysis, 1 ml of 6 M HCl and 700 μl of water were added to 300 μl of the reaction solution and hydrolyzed for 22 hr at 110°C. After removal of HCl, the amino acids were analyzed in an auto amino acid analyzer (model L5000, Hitachi, Japan).

Purification, Identification and Quantification of Protein-Denaturing Compound. Purification ws performed by HPLC (LC-10A system with a UV–visible detector, Shimadzu, Japan). The substrate fraction prepared as described above was injected on an STR-ODS-H column (150 × 4.6 mm ID, 5 μm; Shimadzu, Japan). The column temperature was 40°C and the flow rate was 1.0 ml/min.

Detection was performed at 280 nm. The gradient was as follows: 0–10 min, 0–70% ethanol in water (linear gradient); 10–15 min, 70% ethanol (isocratic). The fractionation was carried out as follows: fraction 1, 1.01–6.00 min; fraction 2, 6.01–9.50 min; fraction 3, 9.51–10.65 min; fraction 4, 10.66–11.30 min; fraction 5, 11.31–11.80 min; fraction 6, 11.81–15.00 min. Each fraction was dried and dissolved in the same volume of water as that of the original substrate fraction. The protein-denaturing activity of each fraction was assayed as described above. Fraction 4, which appeared to be the active fraction, was again injected on the same column under a different gradient condition for further purification. This time, the gradient was 0–30 min, 0–70% acetonitrile in water (linear gradient). The fractionation was carried out as follows: fraction A, 14.00–17.14 min; fraction B, 17.15–17.99 min; fraction C, 18.00–18.99 min; fraction D, 19.00–22.99 min; fraction E, 23.00–30.00 min. The protein-denaturing activity of each fraction was assayed again, and this time fraction B was the active fraction. Identification of the purified compound in fraction B was performed by FAB-MS, ^{1}H NMR, and ^{13}C NMR. FAB-MS was performed with a Jeol JMX-SX102A mass spectrometer. The ^{1}H and ^{13}C NMR spectra relative to tetramethylsilane were measured with a Bruker ARX-400 spectrometer. The physicochemical data of the purified compound were as follows: FAB-MS *m/z* 541 $[M+H]^+$, *m/z* 563 $[M+Na]^+$, ^{1}H NMR (400 MHz, CD_3OD): δ 1.66 (3H, d, J = 7.1 Hz), 2.44 1H, dd, J = 14.1 and 9.1 Hz), 2.70 (1H, dd, J = 14.1 and 4.4 Hz), 2.76 (2H, t, J = 7.0 Hz), 3.30–3.41 (m), 3.67 (1H, dd, J = 12.0 and 5.3 Hz), 3.71 (3H, s), 3.88 (1H, d, J = 11.5 Hz), 3.97 (1H, dd, J = 9.0 and 4.5 Hz), 4.11 (1H, dt, J = 10.7 and 7.1 Hz), 4.21 (1H, dt, J = 10.7 and 7.0 Hz), 5.91 (1H, br. s), 6.08 (1H, q, J = 6.9 Hz), 6.55 (1H, dd, J = 8.0 and 1.9 Hz), 6.66 (1H, d, J = 1.9 Hz), 6.69 (1H, d, J = 8.0 Hz), 7.51 (1H, s). ^{13}C NMR (100 MHz, CD_3OD): δ 13.52, 31.81, 35.39, 41.26, 51.88, 62.74, 66.87, 71.49, 74.77, 77.96, 78.42, 95.24, 100.92, 109.42, 116.44, 117.06, 121.30, 124.86, 130.54, 130.76, 144.93, 146.24, 155.12, 168.68, 173.19. Quantification of the denaturing compound in the privet leaf was performed by comparing the area of prominent HPLC peak of the compound in the substrate fraction (stable leaf extract) in 280 nm with that of the purified compound.

RESULTS

Subcellular Distribution of Protein-Denaturing Compound and Activating Enzyme in Privet Leaf Cell and Mode of Activation. To assay the protein-denaturing activity of the water extract of privet leaves, ovalbumin was mixed with the water extract of privet leaves. The mixture was applied to SDS-PAGE to examine if intermolecular cross-linkings among protein molecules and de-

naturation occurred. The main band of ovalbumin (Figure 1, lane 1) disappeared when ovalbumin was mixed with the water extract of privet leaves (Figure 1, lane 2), and fuzzy bands appeared in the upper part of the separating gel and at the entrance of the stacking gel. We interpret these data to mean that the water extract of privet leaves exhibited a very high denaturing activity linking proteins into high-molecular-weight aggregates. The amount of lysine in the ovalbumin decreased (60% reduction) specifically compared to other amino acids in ovalbumin (Table 1). Both denaturing activity and lysine-decreasing activity were inhibited when 1% free glycine was added to the reaction solution (Figure 1, lane 3; Table 1).

Several observations suggested that enzymatic activation of the denaturing compound is involved in the denaturing activity of privet leaves, so we prepared two fractions; the first one contains the substrate (denaturing compound) free of the activating enzyme, and the second contains the enzyme free of the substrate (denaturing compound).

The substrate fraction was prepared by homogenizing steamed privet leaves, and it did not show any of the protein-denaturing (Figure 1, lane 4) and lysine-decreasing activity (Table 1) observed in the water extract of unsteamed leaves (Figure 1, lane 2; Table 1). The browning reaction observed in the water extract, which is an indicator of oxidation of phenolics, did not occur in the extract of the steamed leaves, and the color of the extract remained fresh green (data not shown). The browning reaction also did not occur when reducing agents (e.g., 20 mM sodium sulfite, 1% mercaptoethanol) were added to the water extract (data not shown). These facts suggest that an activating enzyme, presumably an oxidase, is involved in the reaction.

The enzyme fraction, which contains organelles such as chloroplasts, also did not show protein-denaturing activity (Figure 1, lane 5) or lysine-decreasing activity (Table 1). This result suggests that the denaturing compound accumulates in the cytosol and that the compound was removed during the washing process of organelles. When the enzyme fraction and the substrate fraction were added together in the reaction solution, strong protein-denaturing activity (Figure 1, lane 6) and lysine-decreasing activity (Table 1) were observed. These strong activities were completely inhibited by the addition of 1% glycine to the solution (Figure 1, lane 7; Table 1), as was also observed in the water extract of privet leaves (Figure 1, lane 3; Table 1).

Purification, Identification and Quantification of Protein-Denaturing Compound. Purification was performed in two steps with HPLC under different gradient conditions. The first step is shown in Figure 2. The substrate fraction was injected on HPLC and the elute was collected in fractions 1–6. Each fraction was dried and redissolved in water, and the protein-denaturing activity against ovalbumin was assayed after activation by the addition of the enzyme fraction.

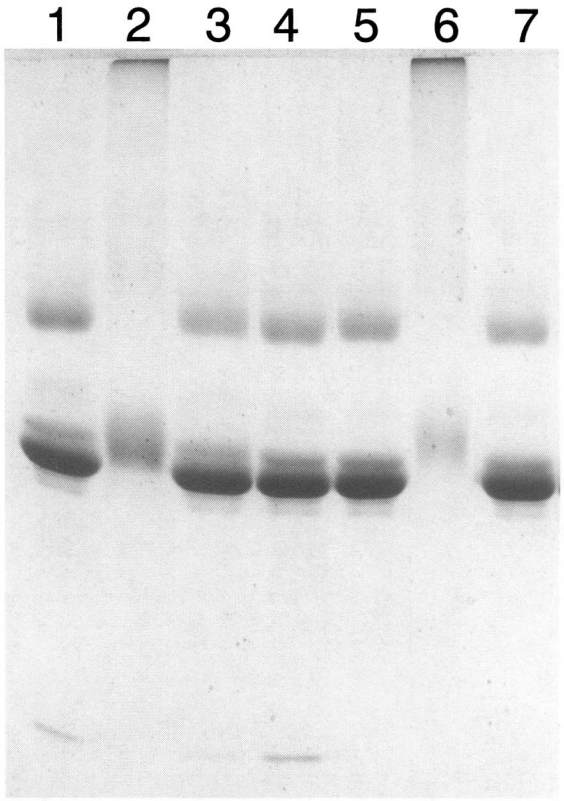


FIG. 1. Effect of protein-denaturing activity of the privet leaf and effect of free glycine on ovalbumin molecule assayed by SDS-PAGE, and compartmentalization of denaturing compound and activating enzyme within a leaf cell. Ovalbumin solution (1%) was treated at 25°C for 2 hr with the water extract of privet leaves, the substrate fraction, the enzyme fraction, or the substrate and the enzyme fraction together, in the presence or absence of 1% glycine. After the treatment, each sample (treated ovalbumin solution) was applied to SDS-PAGE to determine whether high-molecular-weight aggregation was formed by the protein-denaturing activity. Lane 1: control (untreated ovalbumin); lane 2: water extract; lane 3: water extract + 1% glycine; lane 4: substrate fraction; lane 5: enzyme fraction; lane 6: substrate fraction + enzyme fraction; lane 7: substrate fraction + enzyme fraction + 1% glycine. Neither the substrate fraction nor the enzyme fraction exhibited protein-denaturing activity (lanes 4, 5), but when they were added together, a strong protein-denaturing activity was observed (lane 6). This activity was inhibited by 1% glycine.

TABLE 1. EFFECT OF PROTEIN-DENATURING ACTIVITY OF PRIVET LEAF AND EFFECT OF FREE GLYCINE ON AMINO ACID CONTENT[a] OF OVALBUMIN, AND COMPARTMENTALIZATION OF DENATURING COMPOUND AND ACTIVATING ENZYME WITHIN A LEAF CELL

Amino acid	1 Control	2 Water extract[b]	3 Water extract + glycine	4 Substrate fraction[b]	5 Enzyme fraction[b]	6 Substrate fraction + enzyme fraction	7 Substrate fraction + enzyme fraction + glycine
Asp	3.95 ± 0.18[c]	4.02 ± 0.64	4.48 ± 0.29	4.31 ± 0.33	3.98 ± 0.20	3.89 ± 0.50	4.29 ± 0.34
Thr	3.10 ± 0.15	2.83 ± 0.45	3.28 ± 0.15	3.22 ± 0.08	3.18 ± 0.22	2.98 ± 0.35	3.35 ± 0.28
Ser	4.37 ± 0.19	4.07 ± 0.61	4.62 ± 0.16	4.38 ± 0.27	4.42 ± 0.25	4.08 ± 0.37	4.54 ± 0.23
Glu	6.75 ± 0.53	6.39 ± 1.12	7.38 ± 0.56	7.06 ± 0.52	6.71 ± 0.54	6.29 ± 0.74	7.38 ± 0.30
Gly	3.04 ± 0.14	2.90 ± 0.45	—	3.21 ± 0.17	3.09 ± 0.22	3.08 ± 0.57	—
Ala	4.80 ± 0.31	4.27 ± 0.71	—	4.70 ± 0.32	4.70 ± 0.28	4.31 ± 0.60	—
Cys	0.46 ± 0.09	0.28 ± 0.07	0.45 ± 0.06	0.36 ± 0.05	0.43 ± 0.06	0.33 ± 0.06	0.48 ± 0.11
Val	2.61 ± 0.59	2.31 ± 0.32	2.78 ± 0.08	2.46 ± 0.18	2.50 ± 0.28	2.31 ± 0.20	2.57 ± 0.12
Met	2.75 ± 0.11	2.39 ± 0.31	2.80 ± 0.05	2.62 ± 0.06	2.74 ± 0.22	2.55 ± 0.23	2.73 ± 0.31
Ile	1.87 ± 0.42	1.54 ± 0.26	1.85 ± 0.11	1.75 ± 0.14	1.76 ± 0.24	1.46 ± 0.10	1.73 ± 0.11
Leu	3.81 ± 0.44	3.29 ± 0.54	3.92 ± 0.26	3.71 ± 0.23	3.74 ± 0.35	3.25 ± 0.38	3.71 ± 0.29
Tyr	1.30 ± 0.14	1.00 ± 0.14	1.26 ± 0.05	1.17 ± 0.06	1.27 ± 0.20	1.00 ± 0.14	1.18 ± 0.07
Phe	2.24 ± 0.19	2.09 ± 0.32	2.32 ± 0.07	2.17 ± 0.10	2.07 ± 0.12	2.12 ± 0.28	2.16 ± 0.11
Lys	2.92 ± 0.28	1.31 ± 0.34[d]	2.95 ± 0.15	2.96 ± 0.16	2.81 ± 0.18	0.61 ± 0.04[d]	2.80 ± 0.21
His	0.93 ± 0.06	0.75 ± 0.11	0.96 ± 0.02	1.01 ± 0.09	0.95 ± 0.11	0.83 ± 0.23	1.08 ± 0.08
Arg	2.08 ± 0.25	1.84 ± 0.40	2.09 ± 0.02	2.12 ± 0.09	1.94 ± 0.19	1.90 ± 0.24	2.09 ± 0.17

[a] Amino acid content expressed as micromoles per 10 mg sample.
[b] The water extract, the substrate fraction, and the enzyme fraction are described in detail in Methods and Materials.
[c] Values are expressed as mean ± SD of three replications.
[d] Decrease greater than 50% was observed compared with the control sample (untreated ovalbumin).

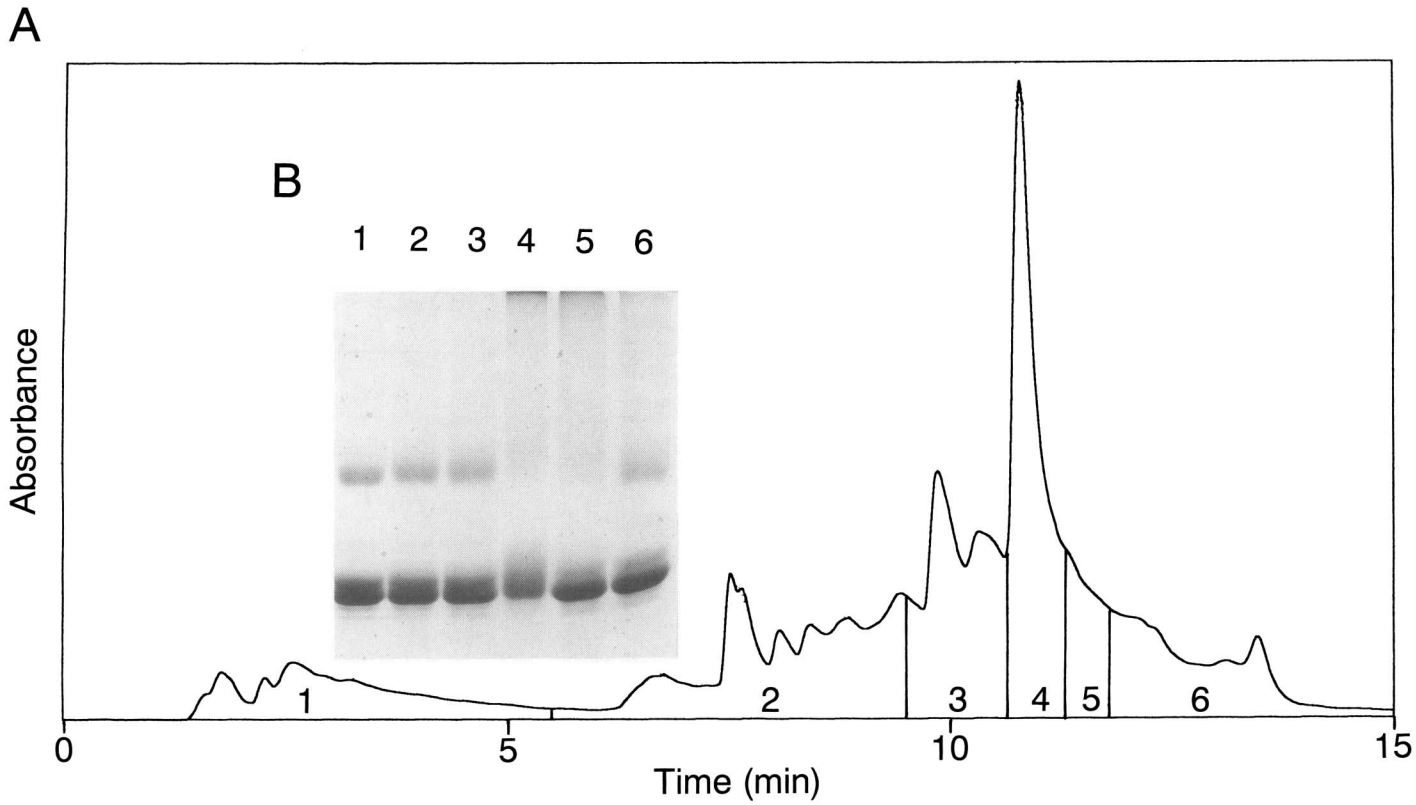
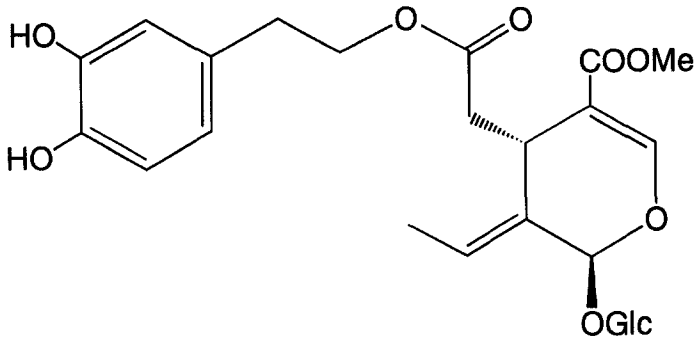


FIG. 2. Purification of the protein-denaturing compound. Purification was performed by HPLC. The substrate fraction was injected on an STR-ODS-H column with gradient of ethanol in water. Detection was performed at 280 nm. The eluate was collected in six fractions (fractions 1–6) (A). Each fraction was dried and dissolved in water, and after activation by addition of the enzyme fraction, the protein-denaturing activity of each fraction against ovalbumin was assayed by SDS-PAGE (B). Fraction 4, which shows a prominent peak, was the active fraction. Fraction 4 was purified further by the same column under different gradient conditions.

Fraction 4, which showed a prominent HPLC peak detected at 280 nm (Figure 2A), was the active fraction (Figure 2B). Fraction 4 was again injected on HPLC under a different gradient condition. Fractionation and assay were performed in the same way and fraction B was the active fraction. Then the identification of fraction B was performed by ^{1}H and ^{13}C NMR. Coincidence of molecular weight and NMR spectra (Kuwajima et al., 1992) showed that fraction B consisted of pure oleuropein (Figure 3), an *o*-dihydroxyphenolic compound that has been reported in several Oleaceae species (Kuwajima et al., 1992; Damtoft et al., 1993). Determination of the oleuropein content in the privet leaf was performed by comparing the area of the prominent HPLC peak at 280 nm (Figure 2A) of the substrate fraction with that of purified oleuropein. The natural concentration of oleuropein in the privet leaf was estimated to be 3.0% of fresh weight.

Comparison of Protein-Denaturing/Lysine-Decreasing Activities in Substrate Fraction, Purified Oleuropein, and Catechol. To determine whether the protein-denaturing/lysine-decreasing activity of the substrate fraction could be associated with the presence of oleuropein, activity of the substrate fraction (which contains 11 mM oleuropein) was compared with that of an 11 mM solution of purified oleuropein after activation by the enzyme fraction. SDS-PAGE and amino acid analysis showed that the 11 mM solution of purified oleuropein has as much protein-denaturing/lysine-decreasing activity as the substrate fraction (Figure 4A, lanes 2,4; Figure 4B). The activity of purified oleuropein was inhibited by 1% glycine (Figure 4A, lane 5) as in the case of the substrate fraction (Figure 4A, lane 3) and the water extract (Figure 1, lane 3). These results strongly suggest that oleuropein is the only major protein-denaturing compound in the privet leaf whose activity is inhibited by glycine.



Oleuropein

FIG. 3. Structure of oleuropein, the protein-denaturing compound in the privet leaf, *Ligustrum obtusifolium* (Oleaceae).

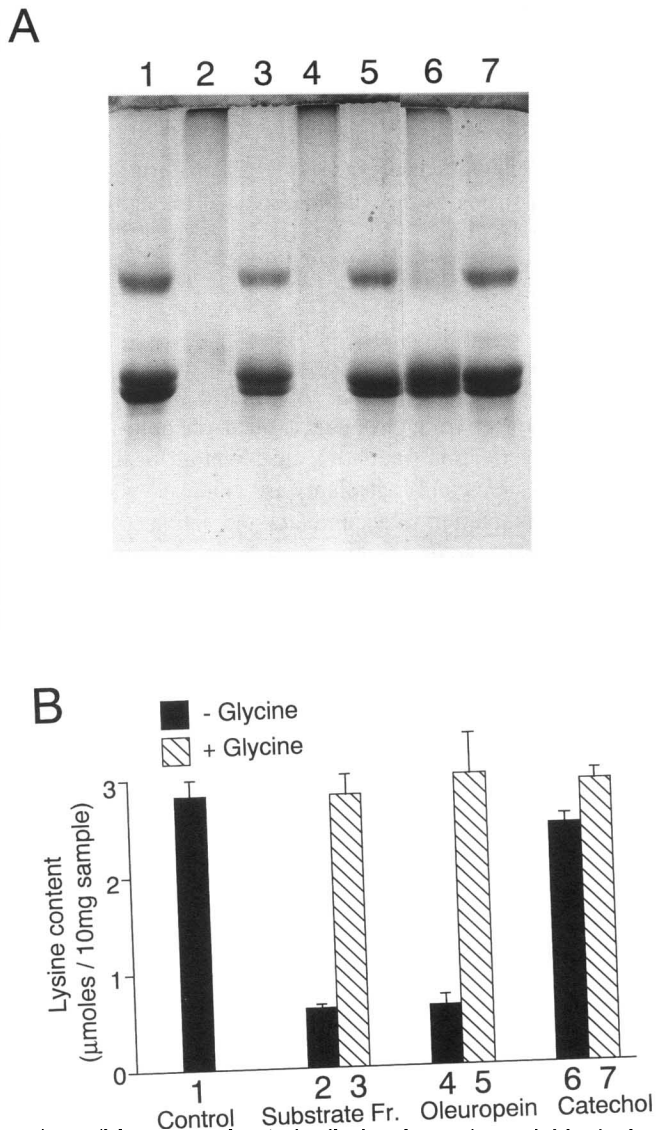


FIG. 4. Comparison of the protein-denaturing/lysine-decreasing activities in the substrate fraction, purified oleuropein, and catechol. After addition of the enzyme fraction, the protein-denaturing activities (A) and the lysine-decreasing activities (B) of the substrate fraction (which contains 11 mM oleuropein), 11 mM purified oleuropein solution, and 11 mM catechol solution were compared in the absence or presence of 1% glycine. 1: control (water); 2: substrate fraction; 3: substrate fraction + 1% glycine; 4: 11 mM oleuropein; 5: 11 mM oleuropein + 1% glycine; 6: 11 mM catechol; 7: 11 mM catechol + 1% glycine. Error bars represent standard deviation ($N = 3$).

To determine whether the *o*-dihydroxyphenolic structure is related to the denaturing activity of oleuropein, catechol (i.e., *o*-dihydroxyphenol) was assayed for its denaturing activity. The denaturing activity of the 11 mM catechol solution (Figure 4A, lane 6; Figure 4B) was compared with that of 11 mM oleuropein (Figure 4A, lane 4; Figure 4B) after activation by the enzyme fraction. Catechol activated by the leaf enzyme displayed protein denaturing/lysine-decreasing activity that was not as strong as that of oleuropein. This activity was inhibited by 1% glycine (Figure 4A, lane 7; Figure 4B).

DISCUSSION

This study demonstrates for the first time that a simple molecule such as glycine can act as a protective agent against plant phenolics. This study also reveals that enzymatic activation (oxidation) is essential for the activity of phenolics in some plant systems and that plants are endowed with a sophisticated mechanism of compartmentation to accumulate and activate phenolics.

We showed that the denaturing compound in privet leaf is oleuropein, an *o*-dihydroxyphenolic compound. Oleuropein occurs in large amounts in the privet leaf and the concentration reached 3.0% of fresh weight. Our data clearly show that oleuropein needs enzymatic activation for its denaturing activity. These results are compatible with our chemical model (Figure 5) showing that an *o*-dihydroxyphenolic compound is responsible for the denaturing activity of privet leaf and also with studies that indicate the importance of oxidative activation of *o*-dihydroxyphenolic compounds by oxidative enzymes in plants (Felton et al., 1989, 1992a; Appel, 1993). Enzymatic activation of defense chemicals that are hazardous even to the plants themselves (i.e., oxidized phenolics, hydrogen cyanide, allyl isothiocyanate, etc.) occurs in many plant systems. In these cases, inactive precursors and activation enzymes are compartmentally separated from each other (Jones, 1972; Spencer, 1988; Luthy and Matile, 1984; Chew, 1988; Vaughn et al., 1988; Kowalski et al., 1992). We observed a clear example of compartmentalization in the leaf cells of the privet tree. While oleuropein seems to be localized in the cytosol fraction (including vacuoles), the activating enzyme was found in the organelle fraction, which is enriched with chloroplasts. The privet tree seems to be endowed with an activating mechanism in which chloroplasts are broken by osmotic shock or by other digestive mechanisms only after feeding by herbivores, and this allows the activating enzyme to contact oleuropein. The method we used to determine the localization is indirect and incomplete, and further cytological studies are required to identify in more detail localization of the enzyme and the substrate within a cell.

Although it became clear that oleuropein is the only denaturing compound in the privet leaf, the characteristics of the activating enzyme remain to be

elucidated. As the enzyme fraction has not been purified, it is possible that there is a mixture of several enzymes. Further purification and characterization of the activating enzyme are needed to elucidate the detailed mechanism of denaturation. The denaturing activity of privet leaf resembles closely that of oxidized phenolics. Figure 5 shows our model simulating the chemical mechanism of the phenomena. Dihydroxy phenolics, such as chlorogenic acid, caffeic acid, catechol, and condensed tannins are known to be converted into quinones in the presence of oxidative enzymes such as polyphenol oxidase and peroxidase. These quinones react with the free amino residue of lysine in protein (Pierpoint, 1969; Hurrell et al., 1982; Felton et al., 1989, 1992a; Appel, 1993). Quinones also react with themselves and are known to form brown-colored, high-molecular-weight compounds. As a result, proteins become covalently cross-linked, and high-molecular-weight aggregates are formed (Hurrell et al., 1982). At the same time, the amount of available lysine in the denatured protein is reduced (Hurrell et al., 1982; Felton et al., 1989; Felton and Duffey, 1991a). Aggregation of protein molecules and decrease of available lysine in protein, which are the properties of oxidized dihydroxy phenolics, were observed in our oleuropein solution activated by the enzyme fraction enriched with chloroplasts. Furthermore, strong browning, which has been regarded as a hallmark of phenolic oxidation, occurs in the reaction solution during the denaturing reaction and is completely inhibited by reducing agents such as 20 mM sodium sulfite and 1% mercaptoethanol (data not shown), which we interpret as meaning that the oxidation of phenolics is related to the activating process. Our results are compatible with previous observations that a polyphenol oxidase is localized in thylacoid membranes of chloroplasts (Tolbert, 1973; Golbeck and Cammarata, 1981; Vaughn et al., 1988). The role of polyphenol oxidase in chloroplasts is still obscure, and there has been considerable argument about its possible functions (Vaughn and Duke, 1984; Vaughn et al., 1988; Mayer and Harel, 1979; Mayer, 1987). Our results suggest that in some cases polyphenol oxidase of chloroplasts may play a role in the chemical defense of plants against herbivores.

We have developed a new method to assay the effect of enzymatically activated phenolics based on the fact that phenolics and activating enzymes are compartmentally separated. In other studies, the effects of plant phenolics activated by plant enzymes are often assayed in crude homogenate. As the reaction of oxidized phenolics is an uncontrollable chain reaction, it was difficult to study the properties of oxidized phenolics produced in plant systems. Our simple method, in which phenolics and activating enzyme are prepared individually, enables us to control the reaction process and may be applicable to other studies.

The fact that 1% glycine can completely inhibit the denaturing activity of purified oleuropein and catechol as well as the water extract of privet leaves indicates that free glycine exerts an inhibitory effect on enzymatically activated phenolics. The present data, together with that in previous papers (Konno et

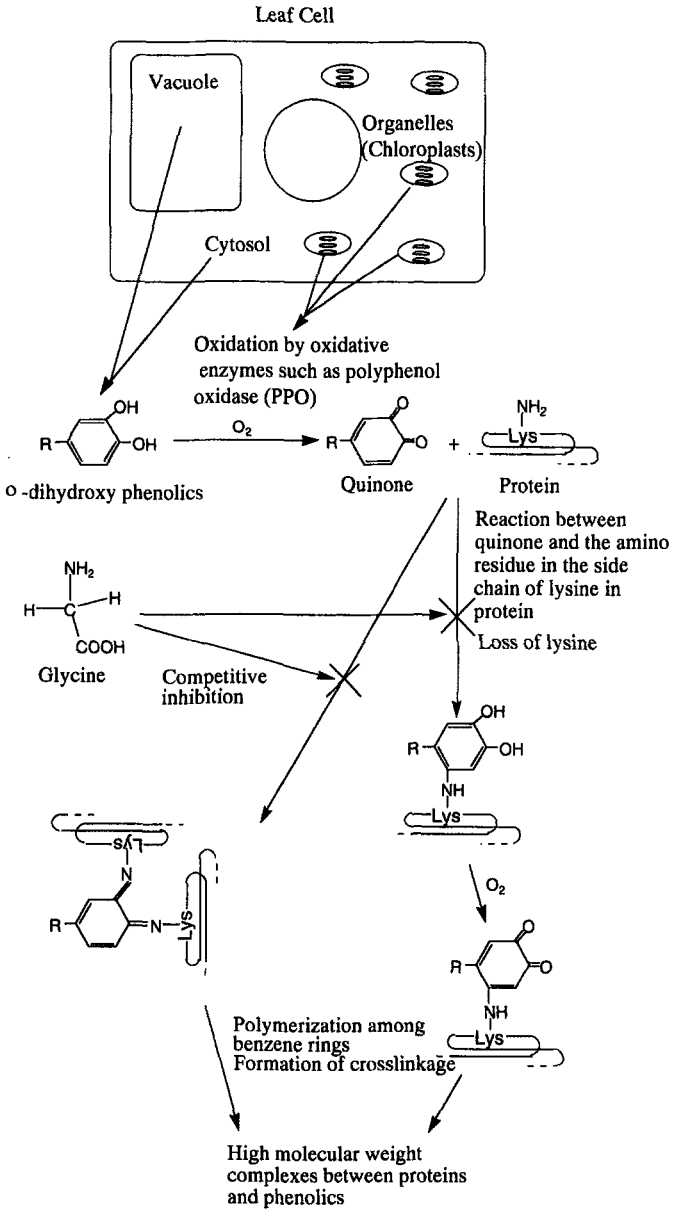


FIG. 5. Proposed chemical model of the protein-denaturing activity of the privet leaf and the inhibitory effect of free glycine. The model considers that (1) oleuropein, which is an *o*-dihydroxyphenolic compound, accumulates in the cytosol or in the vacuoles of the leaf cells, while oxidative enzyme is localized in organelles including chloroplasts;

al., 1996, 1997), suggests that free glycine is secreted by herbivorous insects as an adaptation mechanism to protein-denaturing/lysine-decreasing activity of oxidized phenolics. Although we used 1% free glycine to inhibit protein-denaturing activity, the concentrations of glycine in the regurgitated digestive juices of some privet specialists was only about 0.4% (Konno et al., 1997). In silkworms (*Bombyx mori*) fed an artificial diet to avoid effects from leaves, the concentration of glycine was highest in the anterior part of the midgut, and glycine concentration there was approximately twice that observed in the regurgitated digestive juice (Konno et al., 1996). The in vivo concentration may be about 1%, but 0.4% glycine can also inhibit the denaturing activity (data not shown). Still, our study is an in vitro one, and uncertainty remains about the physiochemical conditions (e.g., glycine concentration, redox conditions) and the timing of mixing in vivo, which can alter consequences. Further studies with other approaches are needed to conclude that glycine is important in vivo.

The amino group of free glycine is known to react with oxidized phenolics (Pierpoint, 1969) in the same manner as the free amino group in the side chain of lysine (Pierpoint, 1969; Hurrell et al., 1982; Felton et al., 1992a). We hypothesize that the amino group of free glycine competes with the free amino group of lysine in proteins for interaction with oxidized phenolics. Other free amino acids may be used by some herbivores as counteractive agents against the denaturing activity of oxidized phenolics. In our model glycine is assumed to be lost by binding to oxidized phenolics, but this loss is less detrimental for herbivores than the loss of lysine, which is an essential and more valuable amino acid.

Species from different families that feed on the privet tree appear to use glycine against the protein-denaturing activity of phenolics. Although not as abundant as in *Brahmaea wallichii* and *Dolbina tancrei*, free glycine is found in a wide range of Lepidoptera larvae (Konno et al., 1997). As oxidized phenolics oocur in a wide variety of plant species, it is likely that glycine secretion and similar mechanisms used to counteract the impact of oxidized phenolics might occur more generally in herbivores.

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(2) during the feeding process by herbivores, compartmentalization is broken, and oleuropein is converted into a quinone by oxidative enzyme; (3) the quinone covalently binds to the amino residue in the side chain of lysine and causes protein denaturation and decrease of the amount of lysine in the protein; and (4) the amino residue of free glycine inhibits protein-denaturing activity of oxidized phenolics of the privet leaf in a competitive manner.

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ATTRACTION OF FEMALE AND MALE *Bactrocera papayae* TO CONSPECIFIC MALES FED WITH METHYL EUGENOL AND ATTRACTION OF FEMALES TO MALE SEX PHEROMONE COMPONENTS

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Abstract—The attraction of female and male *Bactrocera papayae* to conspecific males fed with methyl eugenol (ME) and female attraction to male synthetic sex pheromone, *trans*-coniferyl alcohol (CF), were evaluated in a wind tunnel. Earlier and greater attraction were exhibited by both females and males to ME-fed than to non-ME-fed males as dusk approaches. Males increased their precopulatory behavior (i.e., wing fanning and mounting) during the period of higher attractancy. These data confirm that the consumption of ME enhances the mating competitiveness of males and suggest that ME also functions as a precursor to the male sex and aggregation pheromones. Three phenylpropanoid compounds biosynthesized from ME, coniferyl alcohol, 2-allyl-4,5-dimethoxyphenol, and 3,4-dimethoxycinnamyl alcohol, were detected in male rectal gland along with an endogenous rectal compound, *N*-(3-methylbutyl) acetamide. When offered singly to the females, coniferyl alcohol was found to be most attractive.

Key Words—*Bactocera dorsalis* complex, *Bactrocera papayae*, sex pheromone, coniferyl alcohol, 2-allyl-4,5-dimethoxyphenol, 3,4-dimethoxycinnamyl alcohol, *N*-(3-methylbutyl) acetamide, methyl eugenol, wind tunnel, mating competitiveness.

INTRODUCTION

Bactrocera (*Dacus*) *papayae* Drew & Hancock (Diptera: Tephritidae) previously known as *B. dorsalis* (Mal B) is a member of the Oriental fruit fly,

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B. dorsalis Hendel complex. In this complex, several species are regarded as major or potentially important pests that pose a significant threat to the agricultural industry in subtropical and tropical countries. In Malaysia, *B. papayae* is a serious pest infesting many commercially grown fruits (Tan and Lee, 1982).

In order to control these fruit fly species, methyl eugenol (ME), a highly potent male attractant, was extensively used with great success especially in male annihilation programs (Steiner et al., 1965). Recently, it was found that the consumption of ME enhances the mating competitiveness of males (Shelly and Dewire, 1994; Tan and Nishida, 1996).

Chemical analyses showed that *B. papayae* males converted ME to 2-allyl-4,5-dimethoxyphenol (allyl-DMP), which accumulated in larger amounts, and to relatively smaller amounts of *cis*- and *trans*-coniferyl alcohol and *cis*-3,4-dimethoxycinnamyl alcohol (*cis*-DMC). These compounds were found in the rectal gland (Nishida et al., 1988a,b; Tan and Nishida, 1996). An amide compound, *N*-(3-methylbutyl) acetamide (*N*-MBA), was detected in trace amounts and reported as a minute volatile component of the rectal gland (Perkins et al., 1990). *trans*-Coniferyl alcohol (CF) has been identified as a male sex pheromone (Tan and Nishida, 1996). It was found to attract conspecific females at close range and stimulated ovipositor extrusion at the point of highest concentration. Similarly, at close range males were also strongly attracted to and feed on the compound (Tan and Nishida, 1996). The rectal compounds were released into the air during dusk, which coincided with the period of fruit fly courtship (Nishida et al., 1988a).

In light of the possibility of using sex pheromones as a tool for monitoring, surveillance, and control of female fruit flies, information on the attractancy of the male-produced sex pheromone in ME-fed *B. papayae* is still insufficient. Here, we report on the attraction of female and male *B. papayae* to conspecific males fed with ME, and female attraction to male synthetic sex pheromone and rectal gland compounds.

METHODS AND MATERIALS

Insects. Laboratory-reared *B. papayae* larvae were cultured on an artificial medium containing yeast, sugar, bran, sweet potato, and water. Adult flies were provided with a mixture of water and yeast, protein hydrolysate, and sugar. Males and females were segregated by sex one to three days after emergence (DAE). The flies were maintained under ambient conditions in the insectary on a 12L:12D cycle at 25–29°C and 83–90% relative humidity. Sexually mature virgin males (14–20 DAE) that responded maximally to ME (Tan et al., 1987) and 14–20 DAE females were used for experimentation.

Chemicals. CF and compounds allyl-DMP, *cis*-DMC and *N*-MBA used for chemical attractancy tests were provided by R. Nishida, Kyoto University, Japan.

All the compounds used were pure compounds except *trans*-CF and *cis*-DMC, which were 96.0% *trans* and 97.0% *cis*, respectively. The compounds used for the bioassays were dissolved in absolute ethanol to a concentration of 10 mg/ml.

Live-Fly Pheromone-Source Test. Laboratory bioassays were conducted in a wind tunnel previously described by Jones et al. (1981) and modified by Tan et al. (1987). It is constructed of a transparent polythene tube (0.55 m diam. × 4 m) and has two end grills spaced 2 m apart. Air speed was regulated at 12–15 cm/sec by voltage regulators connected to fans. Fumes of ammonium chloride were used to measure air speed. To prevent possible contamination, the plastic tubing was changed regularly and the grills, wire meshes, and fan blades were thoroughly cleaned with ethanol. Experiments were conducted in a research laboratory that received natural daylight from the south. The temperature in the laboratory was 27–30°C and relative humidity 83–91%. The wind tunnel was placed on a workbench beside the windows.

In the live-fly attractancy tests, 10 sexually mature virgin males were used as a pheromone source. The flies were fed with ME from 08:30 to 09:00 hr during their peak period of response to ME (Tan, 1985) before they were used as test subjects in a wind tunnel in late afternoon and dusk (16:45–19:40 hr). Each male was given 0.5 μl of ME on a Whatman No. 1 filter paper placed on a Petri dish (9 cm diam.), in a laboratory situated ca. 50 m from the insectary. Only males that responded and fed on ME were selected. After consuming ME, the flies were returned to a cylindrical stainless steel cage that had both ends covered with plastic Petri dishes.

Prior to experimentation, sexually mature virgin flies to be used for testing or as a pheromone source (live-fly attractancy tests) were transferred to cylindrical plastic (9 cm diam. × 12 cm) and stainless steel cages (9 cm diam. × 12 cm). Each cage contained food and water. The cages were placed in the research laboratory overnight for the flies to acclimatize to test conditions. Food and water were removed from the cages 30 min before the experiments, and caged ME-fed males were placed in the upwind area of the wind tunnel for acclimatization. The cage was laterally aligned to the upwind grill and was placed on a tripod 20 cm high and 20 cm from the upwind end of the wind tunnel.

In the female attraction studies, 15 sexually mature females were released at the downwind end of the wind tunnel from a cylindrical plastic cage with green mesh (8 × 5 cm) positioned perpendicular to the downwind grill. The cage was supported at 20 cm height by a tripod and situated 1.7 m from the source of attraction. A positive response was recorded when a zigzag flight [zigzagging anemotaxis as defined by Kennedy and Marsh (1974)] over at least 50 cm was performed by a fly to an active upwind section of the tunnel (40 cm from the upwind grill). In the first trial, the females were released at 16:45 hr,

and responses were recorded after 10 min. Subsequently, an interval of 5 min was allowed to collect the released flies. Clean specimen vials (1.0 cm diam. × 5.5 cm) were used to collect the released flies. The procedure was repeated with different flies to avoid habituation. Trials were conducted from 16:45 to 19:30 hr. The average corresponding light intensities in the upwind and downwind area were measured with a lightmeter, ANA-999 (Tokyo Photo-Electric Co. Ltd., Japan). The experiment was replicated three times on different days. Non-ME-fed males were used as a pheromone source in control experiment. The control experiment was conducted separately from the treatment experiment on different days. Male attraction studies were conducted by a procedure that was similar to the procedure described for females, except that caged males were used to assay for their attractancy to sexually mature males instead of females.

Chemical Attractancy Test. Laboratory bioassays of the attraction of sexually mature females to individual compounds of CF, allyl-DMP, *cis*-DMC, and *N*-MBA were conducted before sunset, from 18:15 to 19:15 hr. For each of the bioassays, 3 μl of the solution (concentration = 10 mg/ml in absolute ethanol) were dispensed from a Hamilton 25-μl syringe on a Whatman No. 1 filter paper (4.25 cm diam.) placed on an inverted glass Petri dish that was supported by a tripod 20 cm high and 10 cm from the upwind end of the wind tunnel. Five microliters of pure distilled water were then dispensed on the compound to enhance the evaporation rate. Fifteen sexually mature females were released into the wind tunnel. The chemical tests were conducted in a similar manner to the live-fly pheromone-source tests except that the sample solutions were used as test subjects and after each bioassay the filter paper that contained the chemical was discarded and the glass petri dish thoroughly cleaned with ethanol to prevent contamination. As controls, the tests were repeated with absolute ethanol in place of the sample solutions. During experimentations, lighting was maintained at ca. 34 lux by fluorescent lights to facilitate measurement and dispensing of the sample solutions. Six replications of the experiment were performed.

Percentages were transformed to a modified arc-sine square root and were then subjected to analysis of variance. Means were separated by Student's *t* test and Duncan's multiple range test.

RESULTS

Live-Fly Pheromone-Source Test. Our results show that ME-fed males used as the pheromone source attracted more conspecific flies of both sexes than males not fed with ME ($P < 0.01$) (Figures 1 and 2). When females were tested against conspecific ME-fed males, the peak attractancy was observed to

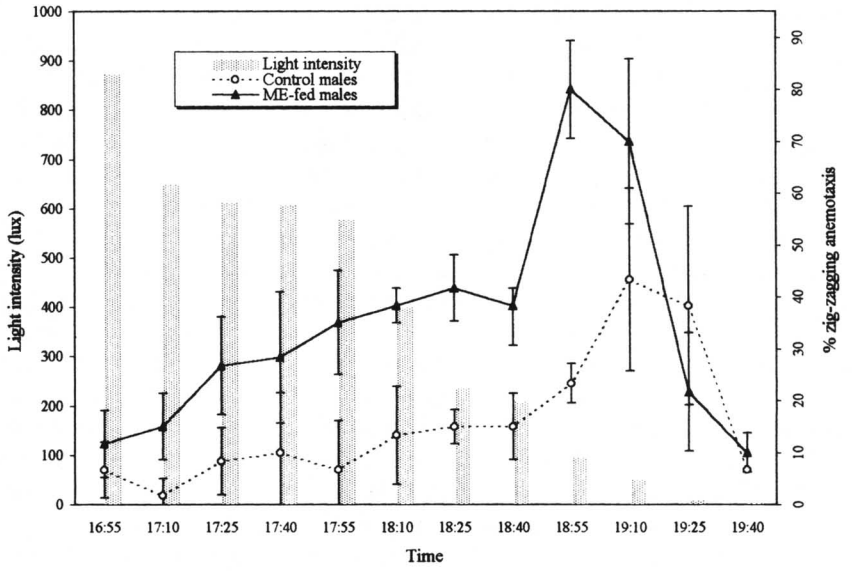

FIG. 1. Mean response of female *Bactrocera papayae* to caged conspecific males in a wind tunnel; four replicates. Vertical bars represent standard errors; comparison of means with Student's t test ($P = 0.01$).

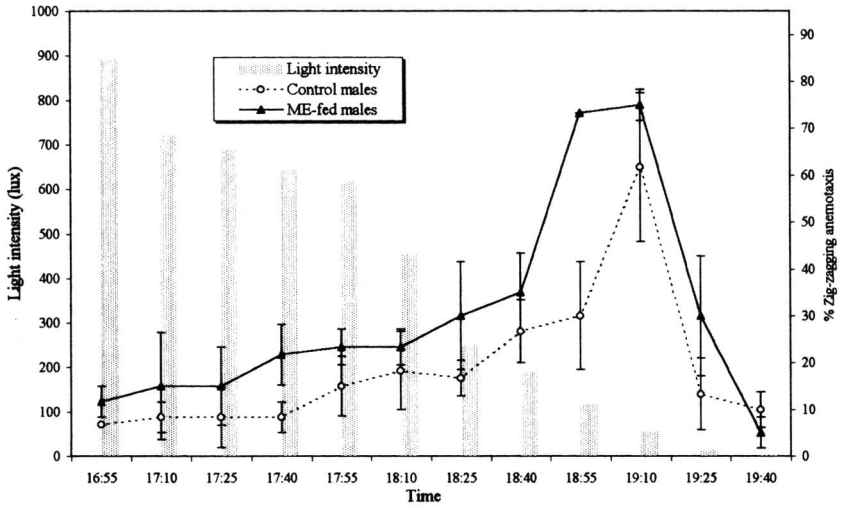

FIG. 2. Mean response of male *Bactrocera papayae* to caged conspecific males in a wind tunnel; four replicates. Vertical bars represent standard errors; comparison of means with Student's t test ($P = 0.01$).

occur earlier (at 18:55 hr; ca. 40 min before sunset), under average light intensity of 117 lux compared with responses of females to control males, which peaked at 19:10 hr under light intensity of 55 lux (Figure 1). Maximal responses of females to ME-fed and control males decreased after a drop of ca. 40–60 lux in light intensity. When light intensity was less than ca. 10 lux, females did not exhibit higher percentages of response to ME-fed males ($0.20 < P < 0.25$). Similarly, when responses of males to conspecific males were assayed (Figure 2), ME-fed males attracted higher percentages of males, with a peak occurring at approximately 18:55 hr under an average light intensity of 94 lux, in comparison to the attractancy of males to control males, which peaked at 19:10 hr at a light intensity of 51 lux. Subsequently, a drop of ca. 40 lux corresponded to a decrease in the responses of males to conspecfic ME-fed and control males. As the light intensity decreased to less than 10 lux, males also showed no significant difference ($0.30 < P < 0.40$) between the attractancy to conspecific ME-fed and control males (Figure 2). ME-fed males were equally attractive to males and females (Figure 1 and 2). Similar results were also observed when males and females were compared for responses to conspecific control males.

Observations of the caged ME-fed and control males used as pheromone sources revealed that peak attraction responses of males and females corresponded with the calling activity in the cages. Approximately 50% of the caged males moved and flew rapidly in an erratic manner. This increase in the locomotor behavior was also accompanied by an increase in physical contact, where males would often engage in head-on collisions and "boxing" behavior. Such displays were observed when males exhibited aggressiveness in defending their individual territories. Males engaged in wing fanning and attempts to mount and copulate with other males. Two types of wing fanning were observed: one type was just prior to mounting accompanied by the rhythmic beating of the abdomen by the rear legs; and the second type occurred as the male settled onto the back of the other male in mounting attempts. Released flies that responded to the sexually excited males would orient to the source and perform zigzagging anemotaxis. Upon locating the source, the flies would either land on the cage, the sides of the polythene tube, or the mesh of the upwind grill. We also observed that some females extruded their ovipositors after landing on the cage containing ME-fed males. However, no copulations between the caged control males and released conspecific females occurred.

In the male attractancy to conspecific males, released males were also observed to fan their wings and a small number (ca. 10%) attempted to mount each other. When light intensity decreased to less than 50 lux, the mating activity of the caged ME-fed and control males declined rapidly. The caged males remained passive and displayed only occasional locomotion behavior.

Chemical Attractancy Tests. The response of virgin females of CF shows

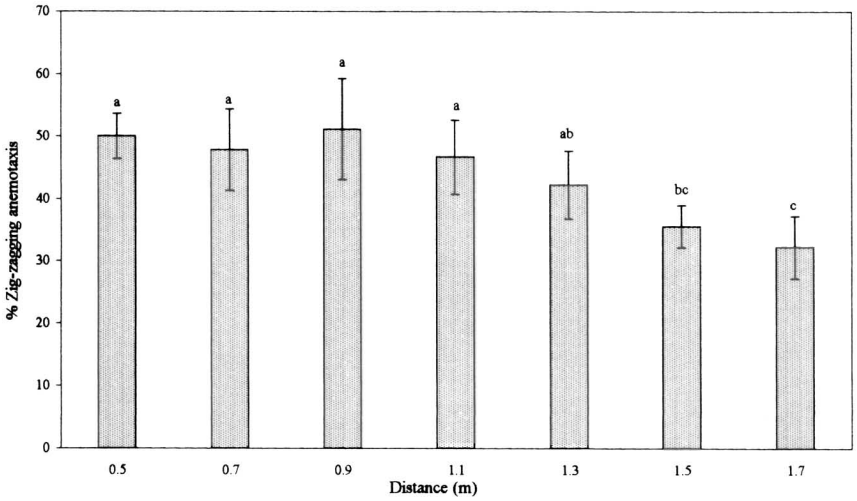

FIG. 3. Mean response of female *Bactrocera papayae* to *trans*-coniferyl alcohol (CF) placed at different distances in a wind tunnel; six replicates: Vertical bars represent standard errors, bars having the same letter are not significantly different at $P = 0.01$; comparison of means by Duncan's multiple-range test.

that over 40% responded by zigzag anemotaxis to the source when released from a distance of less than 1.3 m (Figure 3). When CF was placed at distances between 0.5 and 1.1 m, it attracted significantly higher percentages of females compared with distances more than 1.5 m ($P < 0.01$) (Figure 3). Female responses to individual compounds of the male rectal glands are compared in Figure 4. ME-fed and control males (at peak response times) attracted significantly more conspecific females than were attracted to individual compounds of CF, allyl-DMP, *cis*-DMC, and *N*-MBA ($P < 0.01$) (data obtained from Figure 1). Attraction of virgin females to CF, allyl-DMP, *cis*-DMC, and *N*-MBA was low, with less than 20% of flies responding by zigzagging anemotaxis. Of all the individual compounds tested, CF was shown to be most attractive to the females.

In all the chemical attractancy tests, only a small number (ca. 1%) of females actually landed on the source. However, sexually receptive females would respond by zigzagging anemotaxis to the active upwind section. The flies appeared to have flown against the wind to the active section by locating the pheromone plume. Upon missing the plume, the flies would normally land on the sides of the polythene tubing.

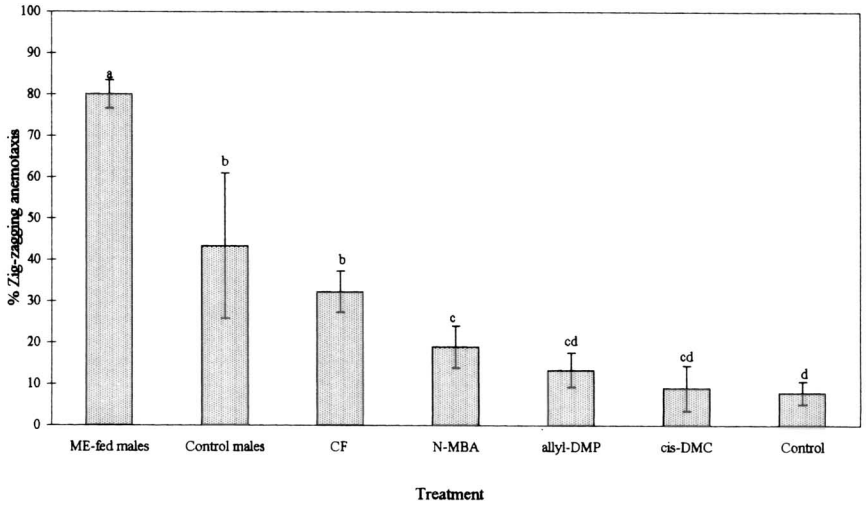

FIG. 4. Mean response of female *Bactrocera papayae* to conspecific males and various chemicals placed 1.7 m from the flies in the wind tunnel; six replicates. Data on ME-fed and control males, respectively, obtained from Figure 1. Absolute ethanol was used as control. Vertical bars represent standard errors, bars having the same letter are not significantly different at $P = 0.01$; comparison of means by Duncan's multiple-range test.

DISCUSSION

ME is a precursor to male sex and aggregation pheromones in *B. papayae* (Tan and Nishida, 1996). Similar results were also obtained through chemical analyses and behavioral studies on the Oriental fruit fly, *B. dorsalis*, and another species in the *B. dorsalis* complex, *B. carambolae* (Tan and Nishida, 1996). Phenylpropanoids, CF, allyl-DMP, and *cis*-DMC, are male attractants to *B. papayae* and *B. dorsalis* (Nishida et al., 1988a; Tan and Nishida, 1996; Tan, 1996). Allyl-DMP was most attractive, and *cis*-DMP least attractive when presented to the males (Tan, 1996). The phenylpropanoids are accumulated in the male rectal gland (Nishida et al., 1988a). Compounds CF and allyl-DMP were immediately detected in the rectal gland after males consumed ME. In our study, the phenylpropanoids were released 10 hr after the males consumed ME, which corresponded to the period when ME-fed males engaged in wing-fanning and mounting. Our results suggest that *B. papayae* males release the pheromone by wing fanning before and during mounting attempts to copulate. Decreasing light intensity as dusk approaches is regarded as the normal stimulus required for the flies to initiate mating behavior. Observations of ME-fed males in a field cage

(2.1 × 2.1 × 2.1 m) showed that virgin females were attracted to and fed on the rectal gland secretion of males in copula with other females (unpublished data).

The mating behavior of caged males used as a pheromone source resembles that of *B. dorsalis* (Kobayashi et al., 1978). *B. papayae* males utilize a lek mating system, a communal display area where males congregate to attract and court females for the sole purpose of copulation (Emlen and Oring, 1977). Lek formation in the *B. dorsalis* complex has been proposed as a form of distraction by the male when releasing the sex pheromone, which simultaneously functions as an aggregation pheromone, thereby diverting the attention of other males from a potential female mate (Tan and Nishida, 1996).

Besides acting as a pheromone precursor, ME also confers a significant advantage to males by functioning as an allomone precursor that deters predation by vertebrates. Observations have shown that when sexually mature *B. papayae* were put under stress, the flies ejaculate rectal contents (Tan and Nishida, 1996). A deterrent effect was produced by ME-fed *B. papayae* males on the house lizard, *Hemidactylus frenatus*. In a no-choice experiment, after initial exposure to ME-fed males, the lizards prefer to starve to death rather than feed on the flies (Tan and Nishida, 1992). Studies on the Japanese tree sparrow, *Passer montunus saturatus*, revealed the increasing order of compounds CF < ME < allyl-DMP as feeding deterrent (Nishida and Fukami, 1990). In a choice feeding experiment involving European starlings, *Sturnus vulgaris*, CF was the most potent repellent followed by 3,4-dimethoxycinnamyl benzoate among a group of phenylpropanoids tested (Jakubas et al., 1992). Therefore, in *B. papayae*, it is probable that males also possess an antipredation mechanism through the endogenous production and secretion of an allomone.

There is no previously published report on tephritid male attractancy to male-produced pheromone in a wind tunnel, although male-to-male attraction in the field is known for a number of species. This study has, for the first time, successfully demonstrated the strong response of *B. papayae* males to pheromone from conspecific ME-fed males in laboratory bioassays in a wind tunnel. In the past, the possible reason for the lack of success at proving responses of males to pheromone in the laboratory was attributed to males themselves acting as treatments because they produce pheromone in the bioassay chamber. These unwanted pheromone sources may then compete with the experimental treatments and also convert controls to treatments (Robacker and Hart, 1986). In this wind tunnel study, the pheromone from males is allowed to flow against released flies at a constant rate, thus ensuring that pheromone from the released males does not act as treatment. Clean air that is used to carry the pheromone is delivered from the inlet fan and is drawn out of the tunnel to the outside environment. This further ensures that air delivered into the tunnel is not contaminated.

Consumption of ME enhances the mating competitiveness of males. This is suggested by the strong attraction of females to conspecific ME-fed males in the wind tunnel. In male–male mating competition for virgin females, males that fed on ME performed significantly better (Shelly and Dewire, 1994; Tan and Nishida, 1996). Preliminary results have also shown that ME-fed males achieved earlier peak frequencies in wing fanning and mounting, and more than 50% achieved copulation (unpublished data). Our work showed that females were attracted earlier during dusk to ME-fed males. This probably accounts for the higher mating success in ME-fed males compared with control males of the same cohorts (Tan and Nishida, 1996). Thus, it appears that ME-fed males produced signals that were more attractive. CF alone failed to elicit significantly higher responses of females compared with live ME-fed males. This may be caused by the lack of other minor components.

The ME-fed *B. papayae* male has a multicomponent pheromone system that involves a combination of phenylpropanoids and other rectal compounds. Among the individual compounds tested, CF was the most attractive to virgin females. Our work also showed that for female *B. papayae* in a confined space, CF elicited a significant percentage of zigzag anemotactic flight. When a mixture of phenylpropanoid compounds CF and allyl-DMP was presented to female flies, it elicited higher percentages of response (Nishida, 1996). Therefore, to increase the attractancy of CF, it needs to be blended with allyl-DMP, *cis*-DMC and *N*-MBA in the correct proportions. This requires further investigations.

In the chemical attractancy tests, conspecific females were also attracted to *N*-MBA. In female *B. dorsalis*, *N*-MBA elicited zigzag anemotatic flight (Metcalf, 1990). *N*-MBA was detected in rectal glands of a number of *Bactrocera* spp. (Fletcher and Kitching, 1995). Therefore, it appears that this endogenous rectal amide probably functions as a natural attractant or sex pheromone component that is enhanced by the presence of phenylpropanoids after pharmacophagy of ME.

The fact that conspecific females and males are sexually attracted to ME-fed males suggests the possibility of developing pheromone-based traps attracting both sexes in efforts to improve existing control strategies. Controlled-release formulations of the pheromone components are crucial to ensure their effectiveness in the field. Further work to determine the correct ratios of the pheromone blend is currently being conducted.

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LARVAL BEETLES FORM A DEFENSE FROM RECYCLED HOST-PLANT CHEMICALS DISCHARGED AS FECAL WASTES

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Abstract—Larvae of the leaf-feeding beetles *Neolema sexpunctata* and *Lema trilinea* carry feces on their backs that form shields. We used the generalist predatory ant, *Formica subsericea*, in a bioassay to determine whether shields were a physical barrier or functioned as a chemical defense. Fecal shields protected both species against ant attack. Larvae of both species reared on lettuce produced fecal shields that failed to deter ants. *Commelina communis*, *N. sexpunctata*'s host, lacks noxious secondary compounds but is rich in phytol and fatty acids, metabolites of which become incorporated into the fecal defense. In contrast, the host plant of *L. trilinea*, *Solanum dulcamara*, contains steroidal glycoalkaloids and saponins, whose partially deglycosylated metabolites, together with fatty acids, appear in *Lema* feces. Both beetle species make modifications to host-derived precursors before incorporating the metabolites into shields. Synthetic chemicals identified as shield metabolites were deterrent when applied to baits. This study provides experimental evidence that herbivorous beetles form a chemical defense by the elimination of both primary and secondary host-derived compounds. The use of host-derived compounds in waste-based defenses may be a more widely employed strategy than was hitherto recognized, especially in instances where host plants lack elaborate secondary compounds.

Key Words—*Lema trilinea*, *Solanum dulcamara*, *Neolema sexpunctata*, steroidal glycoalkaloid, larval defense, fecal shield, Chrysomelidae, phytol, palmitic acid.

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INTRODUCTION

The larvae of herbivorous insects, because they are flightless and lack the hard exoskeleton of the adult, are often the most vulnerable stage in the life cycle. Larvae that graze on leaf surfaces are especially at risk from numerous ecological vicissitudes, such as desiccation, plant allelochemicals, and predation (Southwood, 1973; Strong et al., 1984; Bernays and Graham, 1988). When disturbed, many larvae reflexively eject oral fluids or anal effluents. Such enteric emissions, if derived from diets that are chemically noxious, may serve to deter or repel predators (Eisner and Meinwald, 1966; Duffy, 1980; Peterson et al., 1987; Pasteels et al., 1988; Blum et al., 1987; Whitman et al., 1990; Blum, 1994; Olmstead, 1994; Vasconcellos-Neto and Jolivet, 1994; Dickinson, 1996). If the chemical relationships between host, herbivore, and predator are unknown, however, it cannot automatically be assumed that such regurgitants and fecal discharges effectively function as chemical defenses. Experimental verification is necessary to determine whether or not these emissions are incidental by-products of digestion or are adaptations for defense.

The folivorous larvae of shining leaf beetles, members of the subfamily Criocerinae (Coleoptera: Chrysomelidae), have an unusual variation of the enteric-discharge theme: instead of discarding their feces, they conceal themselves beneath a copious mass, or shield, of excrement (Figure 1). Fecal shields are formed by a dorsal anus, which deposits feces on top of the larva, and by rhythmic muscular contractions that then propel feces forward until the larva becomes covered. Shields have long been thought to provide some kind of protection (Bethune, 1909). Putative functions of fecal shields include camouflage, insulation, and predator defense (Olmstead, 1994). For example, morphological appendages, like the urogomphi of larval tortoise beetles (Hispinae) and the larval cases of Camptosomata (Clytrinae, Crytocephalinae, Chlamisinae, and Lamprosomatinae) that include a fecal component, have been found to function primarily as physical barriers to attack (Root and Messina, 1983; Olmstead and Denno, 1993). True chemical defense (toxicity, repellence, or deterrence), although long suspected (Eisner et al., 1967), has not been demonstrated for any leaf beetle species that possesses some kind of waste-based structure or fecal shield system (Pasteels et al., 1988; Blum, 1994; Olmstead, 1994, 1996).

In addition to their unusual shield-forming behavior, larval Criocerinae are also one of only a few herbivorous insect groups to have colonized successfully members of both mono- and dicot plant families. In the New World for instance, members of the genus *Lema* feed almost entirely on plants in the dicotyledenous family Solanaceae. Larvae of the closely related genus *Noelema* feed exclusively on plants in the monocotyledenous family Commelinaceae (Schmitt, 1988; White, 1993). The two plant families are not known to have any secondary compounds in common. Secondary compounds, such as terpenoids, phenolics,

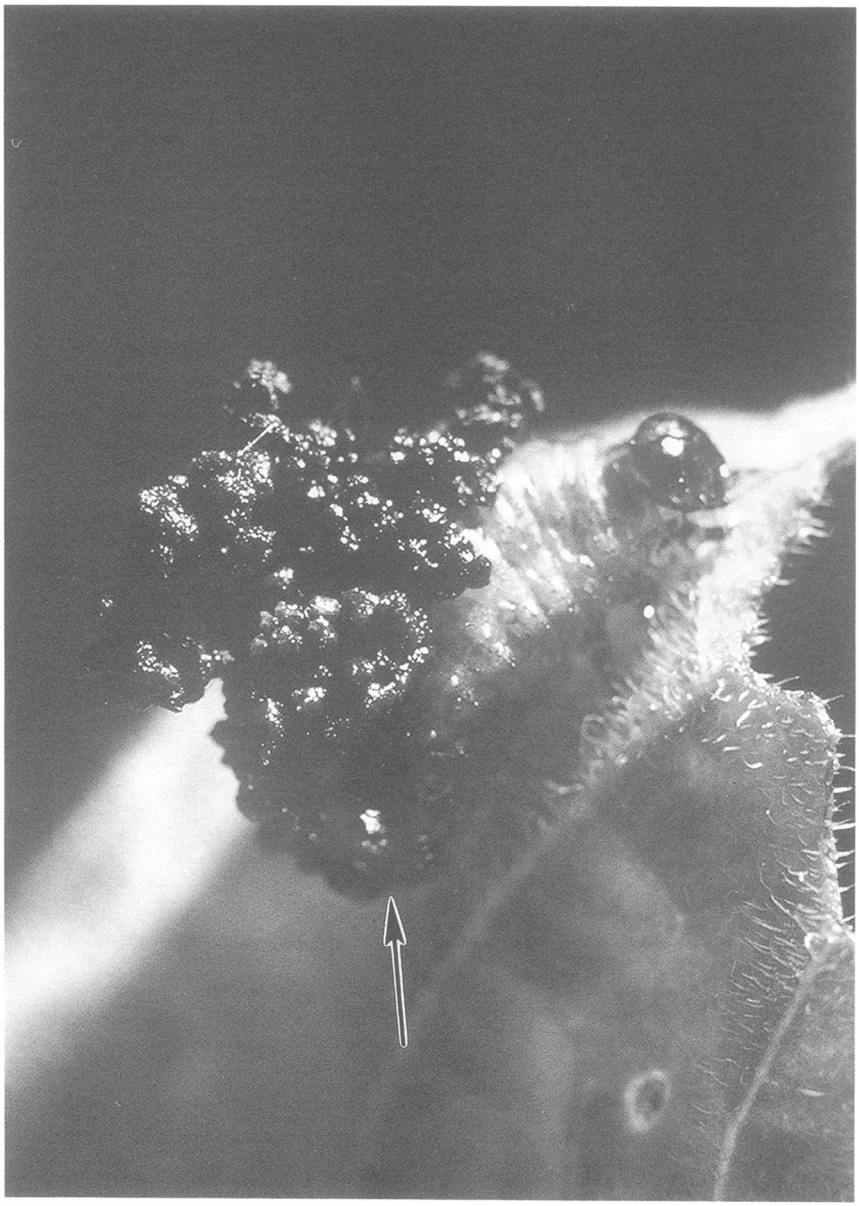


FIG. 1. Larva of the leaf-beetle, *Lema trilinea*, on its host plant, *Solanum dulcamara*. The fecal shield is evident as the glistening mass on top of the larva. The anus (arrow) opens dorsally in the 9th abdominal segment. Peristaltic flexions of the terga convey feces forward to eventually cover the body.

and alkaloids, are often the source of precursors for insect chemical defenses (Eisner and Meinwald, 1966; Whitman et al., 1990 and references therein). The Solanaceae, for example, are well known for the medicinal, antimicrobial, and insecticidal properties of their steroidal glycoalkaloids (SGAs) and steroidal saponins (Evans, 1979; Tingey, 1984; Mitchell and Harrison, 1985; Roddick, 1986; Hegnauer, 1992). In contrast, the Commelinaceae are notable for the lack of complex secondary compounds (Hegnauer, 1992).

In the field, we observed that foraging ants attacking shielded larvae of both genera immediately withdrew, groomed their antennae, and vigorously wiped their mouth parts against the substrate—behaviors indicative of chemical deterrence (Eisner and Meinwald, 1966). We suspected that shields might function in defense against predation and predicted that the defense was a chemical one. We anticipated that *L. trilinea* larvae, feeding on chemically complex hosts, would in some way utilize host-plant secondary compounds. In contrast, *N. sexpunctata* larvae, feeding on hosts lacking complex secondary compounds, conceivably might synthesize their own defensive compounds.

We combined bioassays and chemical analyses to: (1) evaluate the effectiveness of shields as a defense, (2) ascertain whether defensive activity was physical or chemical, (3) locate and characterize any active chemical components, and (4) determine if the defensive compounds were host-derived or synthesized by the larvae.

METHODS AND MATERIALS

Sample Collection. Lema trilinea White feeds on *Solanum dulcamara* L. (Solanaceae) while *N. sexpunctata* Say feeds on *Commelina communis* L. (Commelinaceae). Larvae of both species were collected as second or third instars from wild populations on their respective hosts in Stony Brook, Long Island, New York. *Lema trilinea* larvae were maintained in growth chambers on host cuttings. *Neolema sexpunctata* larvae were reared on potted *C. communis* grown from locally collected seed. Feces for chemical analysis were collected daily and pooled in 2-g aliquots in methanol (MeOH) and refrigerated. Plant samples were collected for chemical analyses from the same plants used to feed beetles.

Lema trilinea and *N. sexpuntata* third instars were field-collected from their respective hosts for use in the bioassay experiments. Larvae had their natural shields removed and were randomly assigned to either a host-diet group or to a diet-modified group. The host-diet culture group, consisting of a total of 71 *L. trilinea* and 89 *N. sexpunctata* larvae were maintained in Petri dishes on cuttings from their respective host plants two to three days before testing. All larvae produced large, viscous shields. The remaining field-collected larvae (100 of each species) were placed in the diet-modified culture group and maintained on

lettuce for two to four days in Petri dishes. Of these, 68% produced normal-appearing shields and were used in the bioassay.

Bioassays. We employed the predatory ant, *Formica subsericea*, in a bioassay to examine the effectiveness of shields and to help direct our search for biological active substances. Predatory ants are important sources of mortality for folivorous beetle larvae (Selman, 1988; Hölldobler and Wilson, 1990; Cox, 1996; Olmstead, 1996; Vencl, personal observation). They are recognized as reliable indicators of the presence of bioactive compounds (Deroe and Pasteels, 1977; Hare and Eisner, 1993; Dyer and Floyd, 1993; Cornelius and Bernays, 1995).

Assays were conducted along foraging trails of the ant at two field sites on Long Island, New York, during June and July of 1993–1996. Assays consisted of exposing beetle larvae to ant attacks. An assay consisted of the placement of a larva (or bait, described below) in an arena formed by a 3-cm × 3-cm wax-coated weighing paper. Each assay was monitored for 5 min or until the test item was taken. An assay was counted if a minimum encounter rate of two ants per minute was observed. In assays with host-derived shields, larvae were induced to regurgitate and eliminate their crop contents before the start of experiments by gently tapping the Petri dish holding the larva. As the droplet of crop fluid appeared, it was immediately soaked up with filter paper, with care taken not to contaminate the shield. This procedure was repeated until the crop contents were depleted, thus limiting the scope of analysis to the feces alone. Crop contents were not emptied and regurgitant not removed during trials with lettuce-reared larvae. Larvae from each treatment group were randomly assayed. Removal and transferal of shields were accomplished with soft forceps, without harm to larvae.

Chemical Fractionation Protocol. The extraction and fractionation protocol, modified from Brown (1987), was designed to remove all organic compounds of moderate to high polarity, deactivate enzymes, and selectively separate the extract into nonpolar, alkaloid (alkaline), and polar fractions. Fresh leaves or feces were weighed and extracted by vigorous maceration (leaf) or sonication (feces) in 10 vol of 80% aqueous MeOH. Extracts were vacuum-filtered and MeOH removed under vacuum at 40°C (a reduction to 1/10th original volume). The resulting aqueous suspension was acidified with an equal volume of 2% H_2SO_4 to produce a pH of ~1.2. The acidic solution was extracted three times with equal volumes of chloroform ($CHCl_3$). The pooled organic solvent was dried with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 25°C to yield a nonpolar, nonalkaloid fraction A. The remaining aqueous phase was made strongly basic (pH 10) by slow addition of concentrated ammonium hydroxide (NH_4OH). The basic solution was extracted with two equal volumes of $CHCl_3$–MeOH (3 : 1) and one to two times with 1.5 vol $CHCl_3$. This second organic phase was dried and reduced in volume as above to yield

a moderately polar, alkaline, and alkaloid-bearing fraction B. The remaining aqueous solution was evaporated to dryness under vacuum at 40°C and extracted with absolute MeOH (three times with filtration) to yield a highly polar extract (C), leaving behind insoluble inorganic salts.

Identification of Shield Compounds: Gas Chromatography–Mass Spectrometry (GC-MS). The nonpolar A fractions of beetle feces, host plants, and lettuce were subjected to GC-MS in an HP-5890 GC equipped with DB-5 column (60 m × 0.32 mm, 25-μm film), temperature programmed for 40°–280°C at 4°C/min, initial hold 5 min, final hold 20 min, 0.5 ml splitless injection, injector 250°C, He carrier at 1.9 ml/min. Peaks were identified by computer searches of the NBS library or the Wiley/NBS Mass Spectral Registry (McLafferty and Stauffer, 1989). Fatty acid (FA) (Sigma No. 189-8; L2376) and phytol (Aldrich No. 13, 991-2) standards were run for comparison on GC-MS.

Identification of Shield Compounds: Fast-Atom Bombardment–Mass Spectrometry (FAB-MS). The alkaloidal B fractions of *L. trilinea* and *S. dulcamara* were analyzed on a Kratos MS 5/90TC mass spectrometer. Samples were dissolved in glycerol and analyzed in positive mode.

Bioassay of Shield Compounds. Baits were used to test chemical fractions and synthetic standards of identified compounds. We used pieces of water-canned tuna with the approximate average size and weight of fourth instars without their shields (mean ± SE: 30.6 ± 4 mg; $N = 22$). Tuna baits were topically coated with fecal fractions, pure compounds identified from feces, or with solvent only (controls). Baits were placed in 5 ml of test solution and the solvent allowed to evaporate in air for 60 min. Ant assays were scored as above. Chemical standards of compounds identified from feces (or the closest commercially available analog) were dissolved in solvent to form 1.0, 0.1, and 0.01% solutions and applied as above.

Data Analysis. Assays of larvae, reported as frequencies (taken as prey or not taken as prey), were analyzed by using model II, 2 × 2 tests of independence and G with the Williams correction (Sokal and Rohlf, 1995). Bioassays of compounds were analyzed with the Mann-Whitney U test (Sokal and Rohlf, 1995).

RESULTS

Shield Bioassays. Larvae of both beetle species with host-derived fecal shields had nearly 100% survival in the ant bioassays (Table 1). After direct contact with shielded larvae, the ants in our assays immediately retreated, began to groom their antennae vigorously, and wipe their mouth parts against the ground. Larvae lacking fecal shields suffered nearly 100% mortality (Table 1).

TABLE 1. ANT BIOASSAY OF *Lema trilinea* AND *Neolema sexpunctata* LARVAE REARED ON NATURAL AND SUBSTITUTE DIETS

Predator response	*L. trilinea*			*N. sexpunctata*		
			Natural diet			
	Host-derived shield	No shield		Host-derived shield	No shield	
Taken	2	33	$P < 0.001^a$	5	39	$P < 0.001^a$
Not taken	33	3		40	5	
			Substitute diet			
	Lettuce-reared shield	Lettuce-reared + transferred shield		Lettuce-reared shield	Lettuce-reared + transferred shield	
Taken	36	2	$P < 0.001^a$	25	2	$P < 0.001^a$
Not taken	0	30		2	24	

[a] Probabilities calculated by 2 × 2 test of independence using G.

Larvae of both species, if restricted to a substitute diet of lettuce, suffered high mortality, even though they were covered by normal-looking shields (Table 1). Deterrence was fully restored to lettuce-reared larvae by mechanical removal of lettuce-derived shields and replacement with host-derived shields transferred from larvae eating their natural diets (Table 1).

Bioassay of Fractions. The actively deterrent constituents of *N. sexpunctata*'s shields are in the nonpolar, A fraction (Figure 2). There was no significant activity in fractions B or C. *Lema trilinea* feces, however, exhibited significant deterrent activity in the A (nonpolar), the B (alkaloid) fraction, and in the polar C fraction (Figure 2).

Identification of Nonpolar Fraction A Compounds. Major nonpolar components of the A fraction from *C. communis* included free phytol, five fatty acids (FAs), and derivatives present mostly as their methyl esters (Figures 3A and 4; Table 2). After passing through the larval gut, the major nonpolar components of *N. sexpunctata* feces were primarily palmitic (hexadecanoic) acid and two new phytol derivatives, 6,10,14-trimethylpentadecan-2-one and 6,10,14-trimethylpentadecan-2-ol (Figure 3B: peaks 2 and 3; Table 2). The two latter

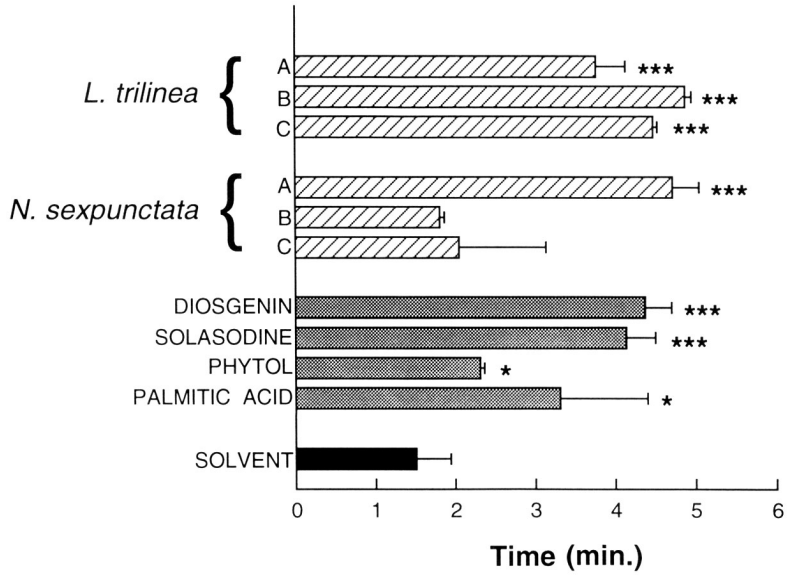


FIG. 2. Ant assay of shield fractions and treated baits. Hatched bars: baits treated with shield fractions A (nonpolar), B (alkaloid), or C (polar). Grey bars: baits treated with pure standards of identified shield compounds. Solid bar: solvent-only control. Baits were coated with 0.1% test solutions; N = 25–30 trials/compound or fraction; error bars = SEM). Probabilities were calculated with the Mann-Whitney U test; significance levels: $*P < 0.05$; $***P < 0.001$.

compounds appear to be formed by modification of phytol. We estimate that the insect accumulates these derivatives on the order of 1.7 times the concentration of the phytol precursor found in the host (0.15 mg/fresh wt feces : 0.093 mg/g fresh wt plant).

The major components of the *S. dulcamara* A fraction were phytol and the methyl ester of palmitic acid (Figure 3C: peaks 4 and 10). *Lema trilinea* feces contained high concentrations of palmitic acid (Figure 3D: peak 5), but not its methyl ester. We also detected three metabolites of diosgenin, including diosgenone and diosgenone minus two hydrogens (Figure 3D: peaks 26 and 28; Figure 4; Table 2). However, the A fraction of *L. trilinea* feces contained only trace amounts of phytol and the phytol metabolites phytanic acid and 6,10,14-trimethylpentadecan-2-one, in addition to small amounts of FAs that were not detected in the host plant. Thus, *L. trilinea* appeared to metabolize phytol almost completely. It was found in feces only in trace amounts, even though it was abundant in its host. The diosgenin aglycone derivatives found in fecal fraction A came from plant glycosides that were found chiefly in fraction C of the host

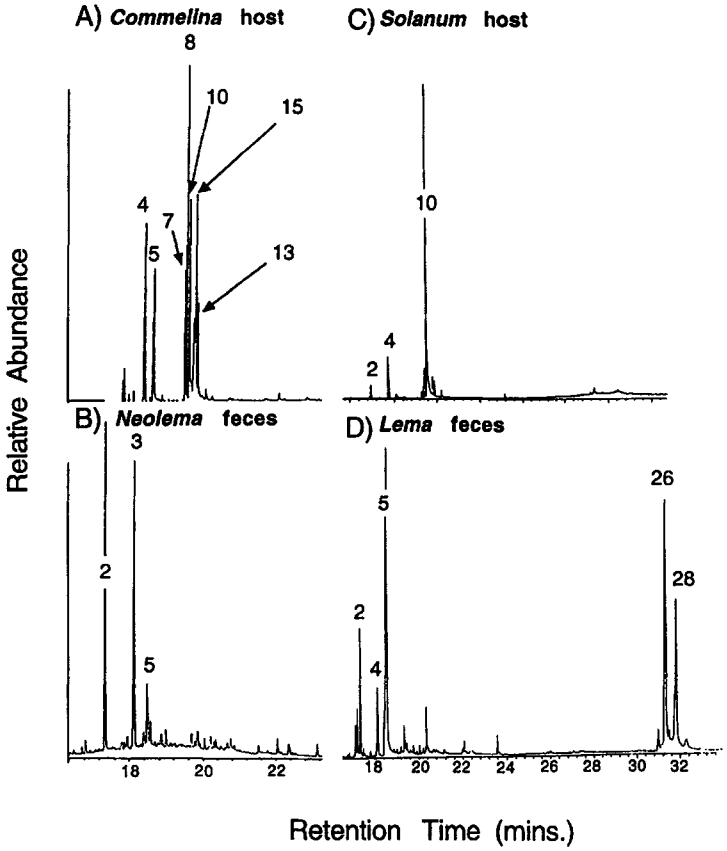
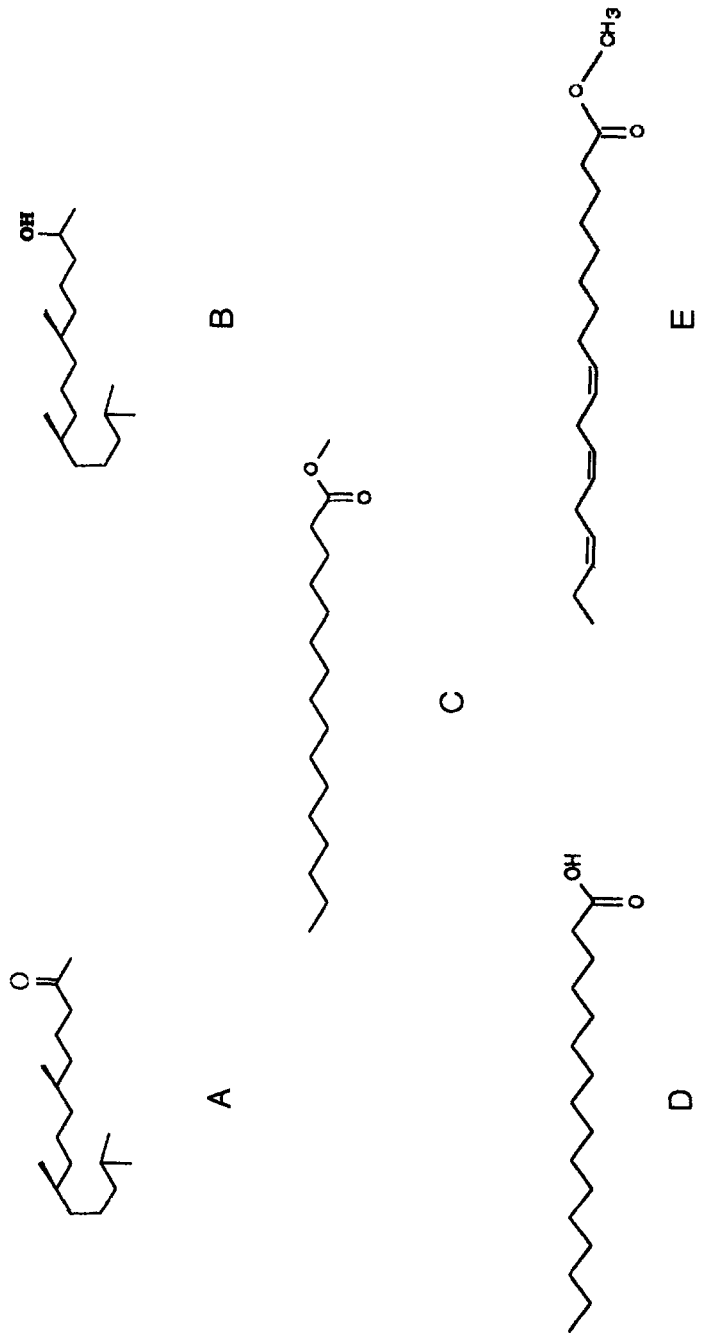


FIG. 3. Comparisons of GC spectra from the nonpolar A fractions. Host-plant input is above (A and C) and the respective larval feces output below (B and D). Peak numbers refer to major compounds (>5% relative abundance) that were characterized by MS and are described in Table 2.

plant. The majority of *L. trilinea*'s fraction A compounds were derivatives of host precursors.

Solanum Alkaloid Fraction and Lema Feces Metabolites. The *L. trilinea* B fraction was highly deterrent (Figure 2). The host FAB-MS ion fragmentation pattern of *S. dulcamara* fraction B corresponds closely to spectra obtained by Price et al. (1985) for solasodine-chacotrios and solasodine-solatrios, which are two steroidal glycoalkaloids (SGAs) found in *S. tuberosum* (Table 3). The host input appeared to be a mixture of two glycosides of solasodine that differed only in the configuration of the trisaccharide moiety. The most informative peaks


A
B
C
D
E

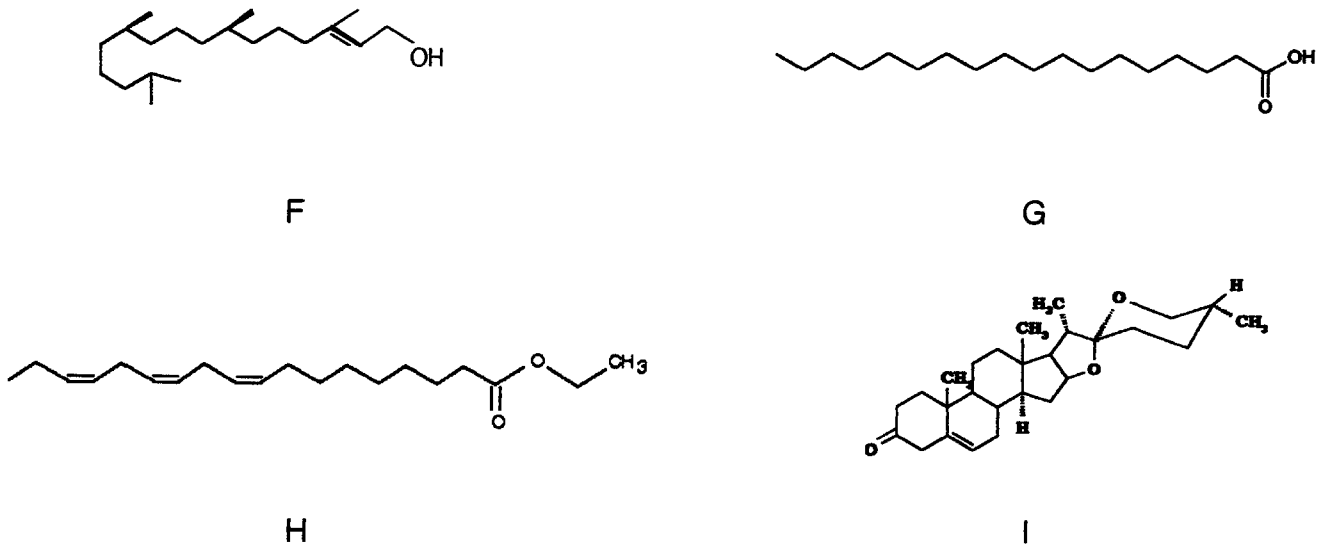


FIG. 4. Molecular structures of fraction A compounds characterized from host plants and fecal shields. A: 6,10,14-Trimethylpentadecan-2-one (peak 2); B: 6,10,14-tri-methylpentadecan-2-ol (peak 3); C: palmitic (hexadecanoic) acid methyl ester (peak 4); D: palmitic (hexadecanoic) acid (peak 5); E: linoleic (octadecadienoic) and linolenic (octadecatrienoic) acid methyl esters (peaks 7 and 8; linolenic acid methyl ester illustrated); F: phytol (peak 10); G: stearic (octadecanoic) acid (peak 13); H: linolenic (octadecatrienoic) acid ethyl ester; I: diosgenone (peaks 26 and 28).

TABLE 2. GC-MS CHARACTERIZATION OF MAJOR FRACTION A COMPONENTS FROM HOST PLANTS AND FECAL SHIELDS

Peak	Retention time (min)	Mol. wt.	Compound	Species system and relative abundance			
				Commelina	*Neolema*	*Solanum*	*Lema*
2	17.4	268	A: 6,10,14-trimethyl-pentadecan-2-one		42.0	[a]	[a]
3	18.1	270	B: 6,10,14-trimethyl-pentadecan-2-ol		41.6		
4	18.2	270	C: methyl palmitate	11.0		10.4	[a]
5	18.5	258	D: palmitic acid	10.6	10.0		27.5
7, 8	19.1-19.5	292-4	E: methyl linoleate + methyl linolenate	22.5		[a]	
10	19.5	296	F: phytol	11.8		72.0	[a]
13	19.75	280	G: stearic acid	7.1			
15	19.8	306	H: ethyl linolenate	15.1			
26, 28	31.0-31.8	412	I: diosgenone				46.0

[a] < 5% abundance.

included the parent ion (MH^+) at 884 and 868 mass units, and the typical solasodine aglycone fragments at *m/z* 396, 412, and 442 (Table 3). In contrast, the *L. trilinea* fraction B showed no indication of any large, glycosylated alkaloid ions. The main spectral component had an *m/z* of 428, which is consistent with a solasodine aglycone to which an additional hydroxyl and one additional double bond have been added by the insect. This metabolite, with its two hydroxyls and two double bonds, was consequently less polar than the plant glycoside, but more polar than the plant aglycone.

FAB-MS of the *L. trilinea* fecal polar C fraction shows that not all of the SGAs were deglycosylated as they passed through the larval gut. Some of the SGA glycosides retained their sugars, which produced peaks 16 mass units higher (at *m/z* 884.4 and 900.7) than the parent SGAs (Figure 5). The most prominent ions relevant to hydroxysolasodine–chacotriose were at *m/z* 884, 720, 430, 428, and 412. The most prominent ions relevant to hydroxysolasodine–solatriose were at *m/z* 900, 738, 458, 430, 428, and 412.

TABLE 3. FAB-MS OF *S. dulcamara* SGAs AND *L. trilinea* FECES

Solasodine-chacotriose[a]		Solasodine-solatriose[a]		*S. dulcamara* fraction B		*L. trilinea* fraction B		*L. trilinea* fraction C	
m/z	RI (%)[b]	*m/z*	RI (%)	*m/z*	RI (%)	*m/z*	RI (%)	*m/z*	RI (%)
								900	54
		884	77	884	6			884	100
868	100			868	30			868	12
		866	4	866	11			866	16
		852	<1	852	2				
850	<1			850	4			860	4
836	<1			836	<1			836	<1
		766	<1	766	<1			766	12
750	6	750	5	750	4				
		738	<1	738	<1			738	16
		737	<1	737	<1				
		736	<1	736	<1				
722	7	722	11	722	6				
721	<1	721	<1	722	2				
		720	5	720	4			720	15
705	<1	705	<1	705	<1			705	4
704	5	704	4	704	5			704	6
632	5			632	<1				
								577	20
								559	18
558	<1			558	<1				
542	<1	542	10	542	<1				
								458	52
442	42	442	35	442	13				
						428	100	428	55
						426	54		
414	31	414	52	414	23				
413	9	413	15	413	8				
412	36	412	45	412	21			412	100
						410	11		
397	31	397	39	397	33			397	52
396	86	396	100	396	100	396	8	396	38
								395	50
						269	13		
						267	8		

[a] From Price et al. (1985).
[b] RI (%) = relative intensity.

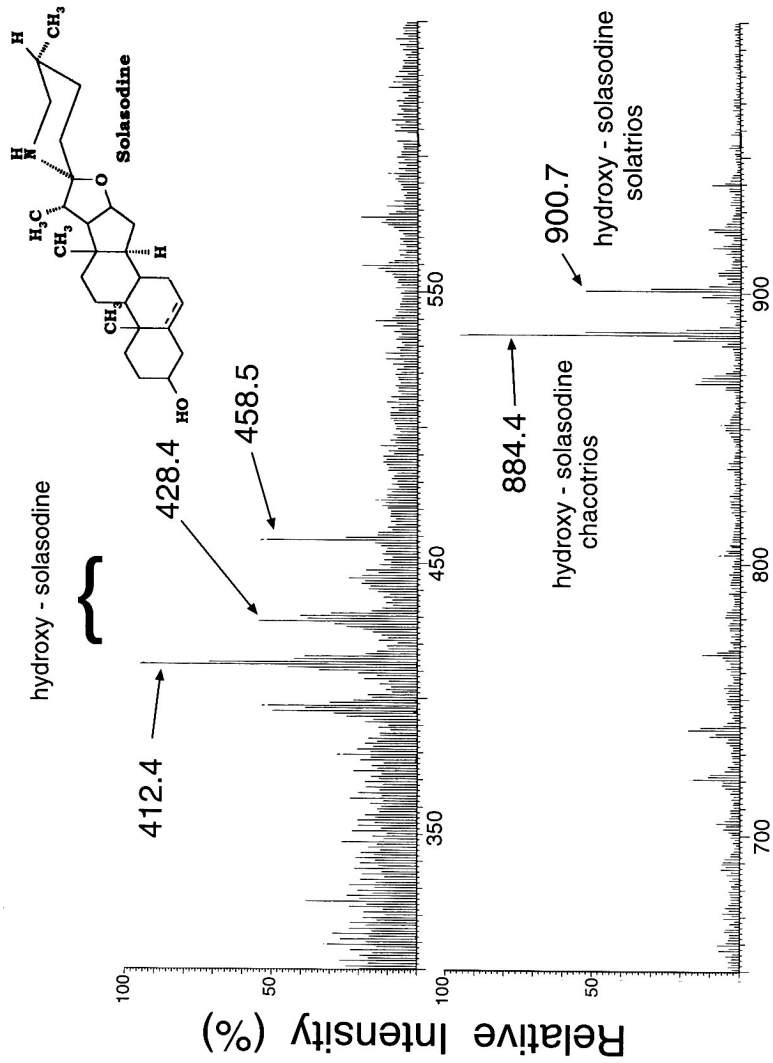


FIG. 5. (Opposite) Positive ion fast-atom bombardment–mass spectrometry of the polar C fraction of *L. trilinea* feces. The two major fragments at *m/z* 884 and 900 are 16 mass units greater than the respective parent solasodine glycosides, and the fragmentation pattern is consistent for two hydroxylated solasodine glycosides. Structure of solasodine is shown in upper right.

Chemical Analysis of Lettuce and Beetle Hemolymph. Lettuce and the hemolymph of 100 *N. sexpunctata* larvae reared on the normal host were analyzed and found to be devoid of any secondary metabolites or any of the primary metabolites we found active in criocerine shields.

Bioassay of Identified Shield Compounds. Pure standards of the major components (or their nearest analogs) identified in both shield systems exhibited significant deterrence with respect to controls (Figure 2). After antennation of the test baits, ants immediately retreated and began to vigorously groom their antennae, and they avoided baits entirely after inspection.

DISCUSSION

This study provides evidence for the defensive efficacy of waste-based shield systems of both *L. trilinea* and *N. sexpunctata*. The consistent rejection of shielded larvae and acceptance of deshielded larvae in the ant assays indicates that the presence of a shield is necessary for protection at least against the generalist predator used in these assays. Based on ant responses after contact with shields, our suspicion that shield defenses had a chemical basis was confirmed. The vulnerability of larvae with diet-modified shields and the restoration of protection to diet-modified larvae with experimentally transferred shields support the hypothesis that shields are defensive adaptations. Furthermore, the active principles of both shield systems appear to be predominantly of dietary origin.

The Commelina–Neolema *System.* Feces of *N. sexpunctata*'s feces consist mostly of phytol derivatives and palmitic (hexadecanoic) acid, and larval defense appears to be based primarily on them. We suspect, based on the structural similarity between phytol and the phytol-like compounds found in feces, that larvae form these putative derivatives by the removal of two carbons from phytol. If so, the limited transformation of phytol would be significant since it might facilitate compartmentalization and elimination of this potential toxin, perhaps by increasing its polarity, and may enhance its deterrent activity. Further research is required to determine the functional significance, if any, of phytol-related compounds. In *N. sexpunctata*'s case, these compounds are evidently more valuable in the feces than they are as energy sources. This may be due in part to the paucity of other defensive candidates in the diet and to the apparent inability of larvae to synthesize their own defensive compounds. The fact that phytol itself exhibits significant deterrence against ants is perhaps the best explanation for the retention of phytol-like compounds in feces.

The Solanum–Lema *System.* In contrast to the simple, phytol-dependent defense of *N. sexpunctata*, both nonpolar and alkaloid fractions exhibited deterrence in *L. trilinea*. Although phytol is the major component of the plant non-

polar fraction, the lack of phytol or its derivatives in the feces indicates that *L. trilinea* can metabolize this molecule completely. This interpretation is supported by the presence in feces of small amounts of phytanic acid, one of the first degradation products of free phytol to CO_2 in vertebrates (Steinberg et al., 1966).

Lema trilinea feces also contain two derivatives of the saponin, diosgenin. Diosgenin, however, was not found in the nonpolar fraction of *S. dulcamara*. It is normally found in plants as a glycoside with a significantly higher polarity and thus should have appeared in the C fraction of our fractionation scheme. However, we were unable to detect diosgenin with FAB-MS in the C fraction. This may have been the consequence of diosgenin having a molecular weight only one mass unit higher than solasodine, and the high abundance of the solasodine ion may have effectively masked diosgenin. We were able to identify it in feces as the aglycone, diosgenone, indicating that the insect deglycosylated it and then added an additional double bond to the diosgenin precursor.

The alkaloid fraction of *L. trilinea* feces was also deterrent in the bioassays. The results of FAB-MS indicate that fecal alkaloids are of dietary origin and that larvae are not passively eliminating these substances but are capable of significantly transforming them. After addition of an hydroxyl to the SGAs, the majority are then deglycosylated and are found in fraction B. It would appear that the final step in the transformation of host SGAs is the addition of a double bond. This must occur, however, after the removal of the three sugars since the FAB-MS showed no sign of fragment ions two mass units less than the hydroxysolasodine trioses.

Successful elimination of natural products as wastes without energy expenditure for their detoxification would be a beneficial strategy, especially if the process yielded a net energetic gain and rendered the compounds more voidable or more bioactive. *Lema trilinea* employs part of this strategy in two instances. Firstly, although the host was found to contain high levels of free phytol, its absence in feces was surprising because the defense of the closely related *N. sexpunctata* is based largely on phytol derivatives. Phytol is largely replaced by steroidal compounds in feces of *L. trilinea*. We hypothesize that *L. trilinea* metabolizes free phytol and the sugar moieties of both SGAs and diosgenin as energy sources. Metabolism of sugar from host precursors leading to incorporation of the aglycone into glandular secretions of larval defenses is a strategy found in the more derived chrysomelid lineages (Smiley et al., 1985; Pasteels et al., 1990). Secondly, palmitic acid is present in relatively higher amounts in feces than in the host. The host contains large amounts of palmitic acid methyl ester. Thus, the most likely source of fecal palmitic acid is by demethylation, although it may be augmented in feces by autogenous synthesis. Our analyses could not definitively distinguish between autogenous versus diet-derived sources of palmitic acid.

Deterrent Properties of Shields. Based on previous studies (Eisner and Meinwald, 1966; Pasteels et al., 1983, 1984), we initially assumed shields would contain volatile repellents that thwart predators before they can damage the soft larval cuticle. The behavior of ants in the bioassays indicated that the defensive compounds identified here were deterrents, rather than repellents: Ants rejected larvae and test baits only after directly inspecting them.

Information is meager about the biological activity, particularly in natural situations, of the compounds we have identified in this study. There is, however, a growing body of evidence that supports the hypothesis that both primary and secondary compounds appear in feces because of their deterrent, rather than their toxic, properties. These two effects may well be concentration-dependent. At high concentrations, for example, primary and secondary metabolites can have toxic effects (Horwitt, 1960; Lawrence et al., 1994; Berenbaum, 1995). At lower concentrations, defensive compounds may act as deterrents or repellents. More important than their possible role as toxins, primary metabolites, such as FAs and phytol, can modulate behavior. Palmitic acid methyl ester for example, was recently shown to deter ants (Eisner et al., 1996; Dani et al., 1996). Several other FAs, including the ones we have identified in shield defenses, evoke necrophoresis (undertaker activity) in predatory ants in the family Formicidae (Blum, 1970). In fact, necrophoresis is elicited at concentrations approximating those seen in this study (Haskins, 1970; Howard and Tschinkel, 1975; Hölldobler and Wilson, 1990). The presence in feces of erstwhile nutrients such as FAs may serve to manipulate ants, perhaps through mimicry of chemical cues that modulate ant house-keeping behavior.

Free phytol and its derivatives require further comment. Free phytol is uncommon in plants. It is usually bound to chlorophyll. As a result, it remained undetected in phytochemical analyses for many years (Sims and Pettus, 1976). Phytol and its derivatives modify feeding and oviposition behavior in several herbivorous insects by directly stimulating sensory neurons that are specifically tuned for their detection (Mohamed et al., 1992; Anderson et al., 1993). Pentadecanone is a defensive secretion in at least one species of formicine ant (Regnier and Wilson, 1968). Recently, reports of unbound phytol have come from a number of plants known for their medicinal properties (Aoki et al., 1982; Rasool et al., 1991; Singh et al., 1991; Pongprayoon et al., 1992; Albini et al., 1994; Brown, 1994) and lend support to the idea that phytol and perhaps its derivatives may well have potent physiological properties, perhaps by synergism with other compounds.

The initial mode of activity producing deterrence may be by mechanisms of cell injury. SGAs and saponins, for instance, have high binding affinities with lipid-bilayer sterols. Sterol binding can disrupt membrane integrity in sensilla of the insect peripheral nervous system (Mitchell and Harrison, 1985; Roddick et al., 1990) as well as in nonneuronal membranes (Appelbaum and

Birk, 1979; Keukens et al., 1996). Additionally, by interfering with acetylcholinesterase function, SGAs can produce secondary toxic effects in the vertebrate peripheral nervous system, and perhaps they do likewise in the CNS of insects where acetylcholine (Ach) is the neurotransmitter (Zitnak, 1977). Thus, these compounds may function in wastes as pharmacological agents.

In conclusion, shields, unlike reflexive discharges, remain continuously deployed to form a standing defense. Although dependent on dietary sources, larvae are capable of completely metabolizing particular host precursors while selectively modifying and eliminating others as active deterrents. The physiological and behavioral evidence support the claim that shield components are deterrents that are incorporated in shields as selective responses to predation, rather than as incidental by-products of digestion and waste elimination. We therefore consider the dorsal anus in these two species to be a specialized delivery system for the conveyance of deterrent substances to a highly strategic location, on top of the larva, where they form a chemical shield.

Our results from a bioassay-guided chemical analysis of the *N. sexpuntata–C. communis* system demonstrated that a diet evidently devoid of elaborate secondary compounds was, nevertheless, an effective source of defensive precursors. Moreover, the active compounds in *N. sexpunctata*'s shield were primary rather than secondary metabolites. In contrast, *L. trilinea*'s defense was based on a mixture of primary and secondary host compounds. We agree with Berenbaum's (1995) suggestion that the role of primary metabolites as allelochemicals mediating plant–herbivorous insect interactions may be greater than presently appreciated, and, as our findings suggest, their importance in herbivorous insect–predator interactions may be underestimated as well. We believe this phenomenon may be a more widespread but overlooked strategy, especially where hosts lack complex secondary chemistry.

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DIFFERENT CAROTENOIDS AND POTENTIAL INFORMATION CONTENT OF RED COLORATION OF MALE THREE-SPINED STICKLEBACK

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Abstract—Female sticklebacks (*Gasterosteus aculeatus*) use the red coloration of males as a criterion for mate choice. Redder males are more attractive. However, males often differ not only in the intensity of their coloration (from dull to bright red) but also in color quality (from yellowish to purple-red). We investigated whether the red coloration of the stickleback is actually a multiple signal made by several pigments. We kept wild caught males singly in tanks until they had built a nest and were ready to accept females. Then, we took standard photographs and measured their colors by spectrometer analyses of the slides and by descriptions of human observers. These two measurements were highly correlated. When analyzing the carotenoid content of the sticklebacks' skin we found two groups of carotenoids (astaxanthin and tunaxanthin/lutein) that were quantified for each individual. The differences in color observed in the fish are correlated to this pigment quantification. Redder fish have more astaxanthin in their skin than yellowish fish, while the color of the yellowish fish appears to be made by tunaxanthin/lutein. Our results suggest that the red coloration of sticklebacks is a multiple trait that is made of at least two different carotenoids. This opens the possibility that male sticklebacks signal more detailed information to females than a one-dimensional trait would allow.

Key Words—Breeding coloration, *Gasterosteus aculeatus*, stickleback, fish, multiple ornaments, signalling, carotenoids, astaxanthin, tunaxanthin, lutein.

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INTRODUCTION

Bright colors are an example of exaggerated signals used for attracting potential mates (Fisher, 1930; Hamilton and Zuk, 1982). According to the "good genes models" of sexual selection (e.g., Hamilton and Zuk, 1982), they are indicators of some quality inherent in an animal that potential partners can secure for their offspring (Anderson, 1994). Such ornamentation can have several facets; accordingly, detailed information could be available in animals with multiple signals (Wedekind, 1992, 1994; Johnstone, 1995).

Sticklebacks (*Gasterosteus aculeatus*) are small freshwater fish with a clear sexual dimorphism during the breeding season, during which males develop a conspicuous red coloration while females remain cryptically colored. Milinski and Bakker (1990) experimentally demonstrated that this red coloration is a trait females use to choose their mate. More intensely colored males are more attractive. This female preference was confirmed in further studies (e.g., Bakker and Milinski, 1991; Milinski and Bakker, 1992; Bakker and Mundwiler, 1994). In natural populations, males differ greatly in their coloration. It is very likely that this is due to costs imposed by the signal (Zahavi, 1975; Grafen, 1990). More intensely colored males are normally in better condition (Milinski and Bakker, 1990; Frischknecht, 1993), suffer less from parasite infection (Milinski and Bakker, 1990), are more aggressive towards conspecifics (Rowland, 1983; Bakker, 1986), but may be more susceptible to predation (Moodie, 1972).

When looking at different males, one gets the impression that their coloration does not only differ in color intensity (from dull to bright) but also in color quality (hue, as defined by wavelength). Some males appear yellow rather than red, while others appear purely red. We have also seen males whose color was between red and deep purple. Therefore, the red coloration of sticklebacks does not seem to be a one-dimensional trait, but a set of multiple traits with the possibility of revealing more detailed information about a male than its health and vigor (Hamilton and Zuk, 1982; Milinski and Bakker, 1990; Frischknecht, 1993). In a first approach to investigate the potential importance of different sorts of red colorations on mate choice in sticklebacks, Baube et al. (1995) used pairs of differently colored male dummies and tested female reaction to them. They discriminated between two kinds of females and analyzed them separately. Low-response females preferred a yellow-bellied dummy over a red-bellied dummy, whereas high-response females displayed no uniform preference between these two color morphs. However, individual preferences of these high-response females could be so different that no uniform pattern is detectable when analyzing these females as a group (see also Discussion).

The red coloration of sticklebacks is due to carotenoids (Brush and Reisman, 1965; Matsuno and Katsuyama, 1976; Czeczuga, 1980). Carotenoids represent a very large group of naturally occurring pigments with various structural

characteristics (Pfander, 1989) and biological activities (Krinsky, 1994). It is not yet entirely clear which carotenoids are stored in the skin of the sticklebacks and are actually relevant as color pigments (the techniques used in the earlier studies do not meet the modern standards of carotenoid identification described in Schiedt and Liaaen-Jensen, 1995). Of high interest in the context of parasite driven sexual selection (Hamilton and Zuk, 1982) is the fact that carotenoids are not only important pigments, but are also essential for the immune system to function properly (e.g., Lozano, 1994). Furthermore, vertebrates cannot synthesize these pigments (Simpson et al., 1981) and depend on the supply in their diet (guppies: Kodric-Brown, 1989; sticklebacks: T. C. M. Bakker, personal communication).

During the breeding season, male sticklebacks may be forced to find the optimal allocation of the carotenoids acquired earlier. On the one hand they need to be attractive to females; on the other hand they should aim at maintaining their health and defending themselves against parasite infection. This compromise might be revealed in the amount of pigments stored in the skin; they are likely to be reflected in the color spectrum visible to females. If so, color would be a multiple signal revealing information about the current needs of the immune system and hence about the susceptibility of a male to specific parasite infections.

The aims of this study were to identify the carotenoid content of stickleback skin fragments and get relative quantification of the different carotenoids of individual fish and to correlate these carotenoid contents to color differences measured by several independent methods. This should allow assessment of whether the red coloration could be seen as multiple ornament. This is also a necessary first step to study the physiology of signaling; it might reveal potential information contained in the most conspicuous sexual trait of sticklebacks, a species for which a wealth of information on mating behavior and mate preference is already available.

METHODS AND MATERIALS

The Sticklebacks. Male and female sticklebacks were caught in a small canal in Roche (near lake at Geneva, Switzerland) and brought to the laboratory. The males were kept individually in 10-liter tanks with some plants (nest material) and gravel and on a 16L : 8D light regime. They were fed with live *Tubifex*, and they were shown ripe females at regular intervals. Those males that subsequently developed their breeding coloration and had built a nest in response to female stimulation were removed from the tank, killed, immediately frozen in a box protected from light, and stored until further use.

Before chemical analyses, the frozen males were photographed in a stan-

dard glass box (see detailed methods in Frischknecht, 1993). Then their skin was removed and used for the chemical analyses.

Identification of Carotenoids. HPLC analysis was performed on a Waters PDA 996 photodiode array system equipped with a Waters 600 multisolvent delivery system pump. The isolated carotenoid esters were quantified by HPLC with Millennium 2010 software (Waters Corporation, Milford Massachusetts) on an analytical Lichrosorb Si column (250 × 4.6 mm) modified with 1% H_3PO_4 in methanol. LC mass spectra were recorded on Micromass VG Platform with negative or positive atmospheric pressure chemical ionization (APCI). The solvent flow was 1 ml/min; corona voltage, negative mode, −2 kV; positive mode, +3.2 kV; cone voltage, −25 V and +20 V; probe temperature, 400°C; source temperature, 120°C.

To identify the main carotenoids, 25 sticklebacks were chosen that could easily be categorized into red ones (10 individuals) and yellowish ones (15 individuals). The skins of similarly colored individuals (red or yellowish) were pooled. Both groups of skins were frozen with liquid N_2, ground, and dried with Na_2SO_4. The samples were washed several times with acetone until the solvent remained colorless. The solvent was removed in vacuo and the oily residue remaining was dissolved in a few drops of chloroform. Each chloroform solution was split into two parts, one of which was saponified (10% KOH in MeOH, room temperature, darkness, argon). Afterwards, *t*-butylmethyl ether (BuOMe) and H_2O were added to the saponified extract, the two phases were separated, and the H_2O phase was extracted several times with *t*-BuOMe. The combined organic phase was dried with Na_2SO_4 and the solvent was removed in vacuo. To remove impurities, the oily residue was washed with DMSO–hexane and afterwards reextracted with *t*-BuOMe. The colored *t*-BuOMe solution was dried with Na_2SO_4 and the solvent evaporated in vacuo. Afterwards, some drops of chloroform were added to the saponified and the nonsaponified samples. After adding acetone–hexane (14:86), the samples were analyzed with HPLC.

HPLC separations were performed with an analytical Lichrosorb Si column (4.6 × 250 mm) modified with 1% H_3PO_4 in methanol for 90 min at a flow rate of 1.2 ml/min. Afterwards, the column was washed for 48 hr with acetone–hexane (14:86) at the previous flow rate. All samples were separated with acetone–hexane (14:86) at a flow rate of 2 ml/min and detected at 470 nm. The separation time was 30 min for saponified samples and 15 min for nonsaponified samples.

Each saponified sample was dissolved in 100 μl acetone–hexane (14:86), and 20 μl was injected into a 20-μl sample loop. The chromatogram of the saponified extract of the red skins exhibited two intense peaks at 5.84 and 22.77 min, and with UV/Vis maxima at 478.7 nm, characteristic for astacene, and at 415.7, 439.9, and 469.0 nm, representing tunaxanthin. Two additional peaks

were observed at 25.54 and 27.02 min, both with UV/Vis maxima at 444.8 and 473.9 nm, and they were identified as lutein and/or its *Z* isomers. The chromatogram of the saponified extract of the yellow skins showed a peak with a retention time of 25.00 min (415.7, 439.9, and 469.0 nm, tunaxanthin), and two additional peaks after 28.26 and 30.2 min (444.8 and 473.9 nm, which were identified as lutein and/or its *Z* isomers). The carotenoids were identified by comparison with authentic reference samples kindly provided by F. Hoffmann-La Roche Ltd., Basel.

Each nonsaponified sample was dissolved in 500 μl acetone–hexane (14:86), and 20 μl was injected into a 20-μl sample loop. The chromatogram of the nonsaponified extract of the red skins showed two intense peaks at 1.51 and 2.08 min, respectively, with UV/Vis maxima of 444.8 and 466.6 nm for the first peak (esters of lutein/tunaxanthin) and 466.6 nm for the second peak (esters of astaxanthin). The chromatogram of the nonsaponified extract of the yellow skins showed an intense peak with a retention time of 1.50 min and a UV/Vis spectrum with wavelengths of 442.3 and 469.0 nm (esters of lutein/tunaxanthin).

Determination of the molecular weights of the saponified samples was performed by HPLC-MS. Due to the high fat content, the molecular weights of the nonsaponified samples could not be determined. For saponified samples of the red skins, molecular weights of 592 (for astacene) at t_R = 10.95 min and 568 (for tunaxanthin or lutein) at t_R = 43–47 min were determined. For the yellow skins the molecular weight was determined as 568 (for tunaxanthin or lutein) at t_R = 43–51 min. A flow rate of 1.2 ml/min was used.

Quantification of Carotenoids. The skin from an individual frozen fish was removed and immediately frozen with liquid N_2, ground, and dried with Na_2SO_4. The mixture was washed several times with acetone until the solvent remained colorless. Organic phases were combined and reduced in vacuo. The oily residue was dissolved in acetone–hexane (14:86) and separated by thin-layer chromatography on MgO with acetone–hexane (14:86). Two bands were observed: a yellow band near the solvent front, and a red one with a low R_f value. In some experiments, an additional weak yellow band in the middle of the other two bands was observed. The bands were cut out, the yellow ones dissolved in acetone–hexane (14:86), and the red one in acetone. The solutions were filtered and the solvent removed in a gentle nitrogen jet.

Each sample was dissolved in 100 μl acetone–hexane (14:86) and 30 μl was injected into the HPLC apparatus (at least two injections per sample). The mobile phase was acetone–hexane (14:86) at a flow rate of 2 ml/min, and the substances were detected at 470 nm. The separation time was 10 min.

As saponification was omitted in the procedure, esters of lutein and tunaxanthin were not separated and only the total amount of these esters was

determined. The combined lutein and tunaxanthin, and the astaxanthin esters were quantified from calibration curves based on standard solutions of lutein ester and astaxanthin ester, respectively.

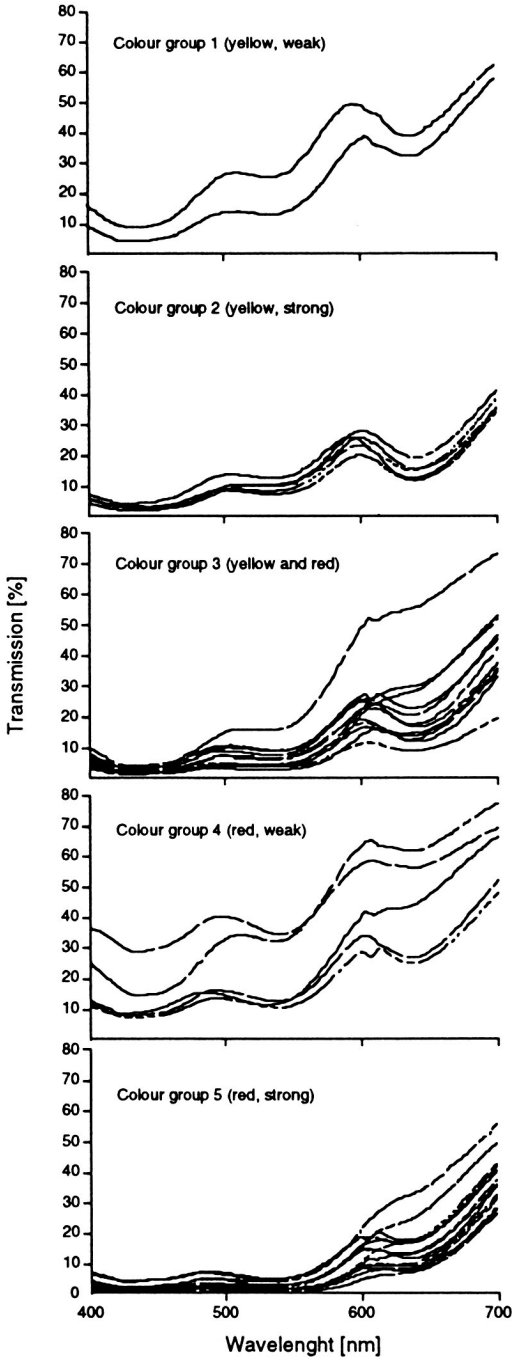
Due to small sample amounts, only relative quantification was possible. This, however, is considered sufficient for discriminating between different groups of color types. In cases where no band was visible in the thin-layer chromatography (for astaxanthin: 13 of 40 fish samples, and for tunaxanthin/lutein: 2 of 40 fish samples), a value of zero was assigned for statistical analyses. This may sometimes be inaccurate, but it at least does not produce a systematic error that could bias our conclusions; rather, it is restricted to increasing the variance within groups.

Measuring Colors by Spectrometer Analysis. For each of the 40 males, an area of about 2 mm^2 of the ventral part of the head was measured from color slides [on Kodachrome 64 film; for method see Frischknecht (1993)], in a Unkon 810 spectrophotometer from Kontron. The transmittance was recorded from 400 to 700 nm, the spectrum visible to humans and to sticklebacks, which have similar cone sensitivities, at 452, 529, and 604 nm (Lythgoe, 1979). Over the wavelength range 320 measurements were taken (Figure 1). This was also done for the white standard color chip (Munsell, white N 9.5) visible on each slide. By setting the white color chip of one particular slide as the baseline reference for 100% transmittance, we calculated a factor describing the percentage of light transmitted by the white color chip on each slide compared to the baseline. The spectrum for each ventral red spot was then multiplied by the inverse. The corrected transmittance for a red spot is given by: corrected $t_{red} = 100/t_{white}$.

By using Endler's (1990) segmentation method, we reduced the spectrum for each fish to a set of values that describes hue (analogous to the dominant wavelength; zero = red end of the visible spectrum), chroma (also called saturation), and brightness (the total area under a spectrum). The four segments for this procedure were each 75 nm long and cover the whole range of 400–700 nm. Since differential brightness due to differences between films and processing are corrected for using the procedure described above, the remaining differences in brightness are inherent properties of the fish's color; therefore brightness in the set of traits was included for describing colors.

Human Description of Colors. For relating color impression to spectrophotometer data, three persons were asked (one of them was author M.F., who was naive at that time with respect to the other data) to judge the slides of the same 40 male sticklebacks. Each saw all slides simultaneously on a light table in front of him/her and ranked them according to redness. In a second round,

FIG. 1. Absorption spectra for the five classes of male sticklebacks (yellow, weak intensity; yellow, bright; yellow-red; red, weak; red, bright) and the carotenoids isolated in their skin, over the wavelength range of 400–700 nm.


Colour group 1 (yellow, weak)
Colour group 2 (yellow, strong)
Colour group 3 (yellow and red)
Colour group 4 (red, weak)
Colour group 5 (red, strong)
Transmission [%]
Wavelenght [nm]
400
500
600
700

they repeated the ranking with black and white slides that were copies of the color slides (Agfa Scale 200 slide film). This way, we reached a score for chroma (saturation) independent of hue. In both rounds, the observers chose one of two methods, whichever suited them best: either assigning each slide a score between 1 (color slides: very dull; black and white slides: light) and 10 (color slides: very bright; black and white slides: intense), or sorting the slides by increasing brightness; this second list was then reduced to the same scale from rank 1 to 10, with fractional ranks. This way, averaging over all three judges was possible.

Since some fish appeared more yellow and others more red in various intensities, two persons (the authors M.F. and C.W., again naive at that time with respect to the other data) created an additional ranking, using the color slides on the same light table to group them on a two-dimensional scale: group 1: mainly yellow, weak intensity; group 2: mainly yellow, strong; group 3: intermediate: yellow and red; group 4: only red, weak; group 5: only red, strong. This assessment as well as the earlier ranking of redness, was independent of the chemical data (the chemical data were not known to the judges at that time).

RESULTS

Color Pigments. The red coloration is made of more than one pigment. We found three different carotenoids in the skin of sticklebacks, identified as astaxanthin, tunaxanthin, and lutein (Figure 2). Since we could not discriminate between tunaxanthin and lutein in the quantification of carotenoid contents per fish skin, these two components have been measured together and the measurements are called hereafter tunaxanthin/lutein. Astaxanthin and tunaxanthin/lutein seem to be independent of each other; the amount of astaxanthin in the skin of the fish did not correlate with the amount of tunaxanthin/lutein ($r = 0.04$, $N = 40$, $P = 0.82$).

Color Measurement versus Carotenoids. The calculation of chroma and brightness from the spectrometer analysis and the ranking of black and white slides by human observers were strongly correlated with each other (ranking vs. chroma: $r = 0.79$; $N = 40$, $P << 0.001$; ranking vs. brightness: $r = -0.82$, $P << 0.001$). The same was true for the calculation of hue from the spectral analysis and the ranking of color slides by humans ($r = -0.94$, $N = 40$, $P << 0.001$). Therefore, the two methods give similar relative quantification of the fish's coloration. The comparison with the chemical analyses show that redder fish have more astaxanthin (compared to hue: $r = -0.31$, $P = 0.05$, two-tailed; compared to human ranking of redness: $r = 0.35$, $P = 0.028$) whereas the content of tunaxanthin/lutein did not significantly correlate with red coloration (compared to hue: $r = -0.07$, $P = 0.67$, two-tailed; compared to human scaling of redness: $r = 0.01$, $P = 0.95$).

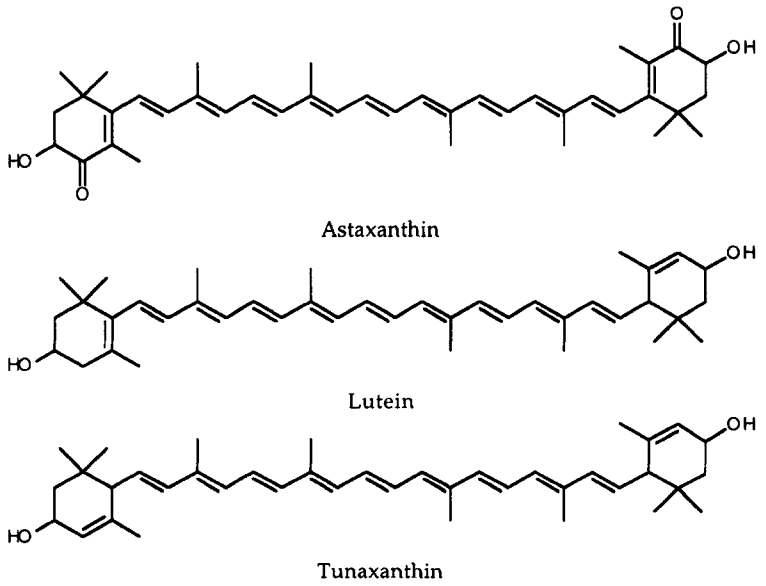


FIG. 2. The chemical structures of astaxanthin, lutein and tunaxanthin (after Matsuno, 1989).

To human observers some fish appeared only yellow, or more yellow than red, while others appeared only red, each in various intensities. The measurements of hue by spectral analysis and the one-dimensional scaling of redness by human observers are not ideal methods to describe these different color patterns. Therefore, two human observers categorized the fish again but this time into five categories of two dimensions simultaneously (see Methods and Materials). Figure 3A shows the values of color measurements by spectrometer of the fish of these groups in relation to the criteria the human observers used for grouping. The five groups differ significantly in chroma, hue, and brightness measured by spectrometer (Figures 1 and 3A and B). A discriminant analysis of these five color groups based on spectrometer measurements reveals that the values for hue and chroma can be used to find a function that distinguishes the five groups ($D = 3.37 \cdot \text{chroma} + 6.14 \cdot \text{hue}$; $F_{\text{global}} = 43.27$, $P << 0.001$) while the values for brightness contain redundant information (difference in r^2 between analyses with two and with three variables < 0.001).

Figure 4 shows the carotenoid content of the fish in these groups again in relation to the criteria used for grouping. These five groups differ in the relative amount of carotenoids (see Figure 4 legend). This seems to result from different amounts of astaxanthin in the groups but not necessarily of tunaxanthin/lutein (statistics in Figure 4 legend). Although the contents of tunaxanthin/lutein them-

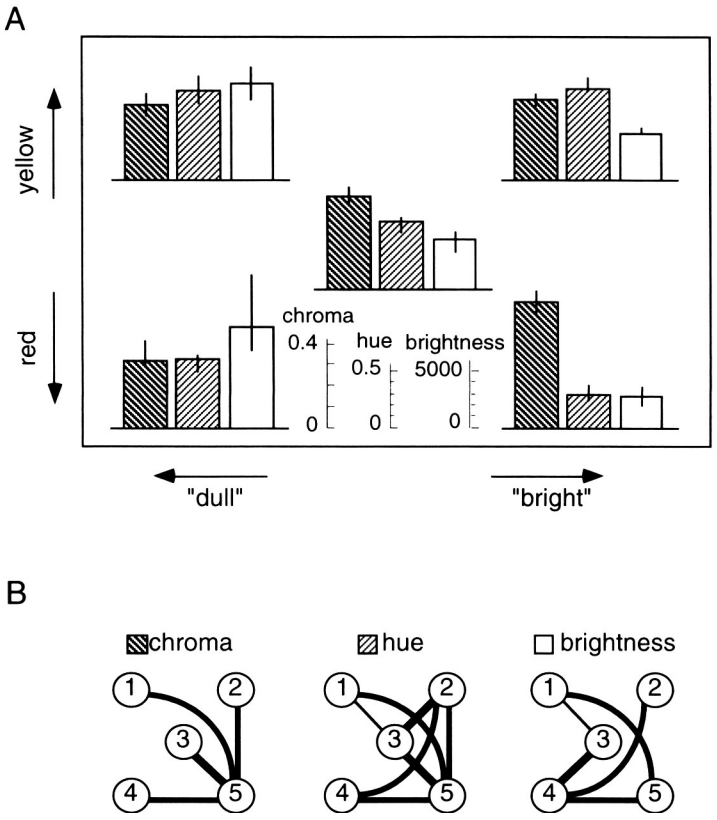


FIG. 3. Spectrometer analyses of stickleback coloration versus two-dimensional description of colors. Photographs of the sticklebacks were categorized by two naive human observers into five groups according to color (red, yellow) and color intensity (dull, bright). (A) Medians and quartiles of measurements of chroma (first bars), hue (second bars), and brightness (third bars) of these fish. In sticklebacks, absence of red color pigments is usually connected with a opaque silver skin that reflects light to a high degree; this usually results in the counterintuitive finding that dull fish have high values of brightness and bright fish, i.e., brightly colored ones, have low values of brightness. The five groups differ in every respect of color measurement (one-way ANOVA, F always > 13.5, $df = 4$, P always $\ll 0.001$, two-tailed). (B) To describe the differences between the five groups in more detail, we performed post hoc analyses (Tukey HSD multiple comparision). Group 1 are the yellow and dull males, group 2 the yellow and bright males, etc. Groups differ from each other when the lines between the group numbers are thick ($P < 0.01$) or thin ($P < 0.05$). No line means no significant difference between groups.

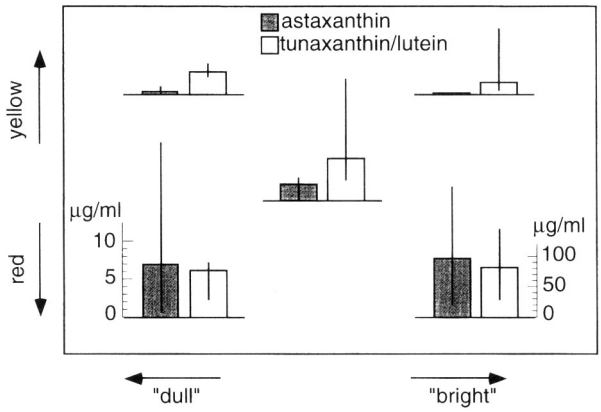


FIG. 4. Carotenoid content of stickleback skins in the five color groups in micrograms per milliliter of hexane–acetone (86:14) solution. Hatched bars give the median ± quartiles of the content of astaxanthin (scale in left corner), and open bars the content of tunaxanthin/lutein (scale in right corner). The groups differ in their relative amounts of carotenoids (percentage astaxanthin of all carotenoids compared between the five groups: Kruskal-Wallis, $df = 4$, $P = 0.043$, two-tailed). This seems to result from different contents of astaxanthin (Kruskal-Wallis, $df = 4$, $P = 0.012$, two-tailed) but not of tunaxanthin/lutein ($P = 0.75$, two-tailed).

selves cannot be used to discriminate yellow and red fish, the highest amounts of this pigment can be observed in some of the yellow fish (Figure 5). When the fish are grouped into those that are yellow or yellowish (group 1–3 pooled) and those that are mostly red in various intensities (group 4 and 5 pooled), we found a discriminant function that significantly discriminates these fish according to their carotenoid content (Figure 5). However, the values for tunaxanthin/lutein and astaxanthin do not correlate significantly with the spectrometer measurements in a canonical correlation (carotenoid contents ranked to take into account extreme values, including brightness: Hotelling-Lawley (H-L) trace = 0.28, $F = 1.56$, $df = 6, 68$, $p = 0.17$; excluding brightness: H-L trace = 0.22, $F = 1.88$, $df = 4, 70$, $p = 0.12$), nor is the relative amount of astaxanthin significantly linked to the spectrometer measurements in a multivariate analysis (including brightness, multiple regression, $F = 1.04$, $df = 3$, $p = 0.39$; excluding brightness: $F = 0.94$, $df = 2$, $p = 0.40$).

DISCUSSION

The breeding coloration of male sticklebacks in our sample is made of at least three carotenoids (two groups quantified here). Absolute amounts varied

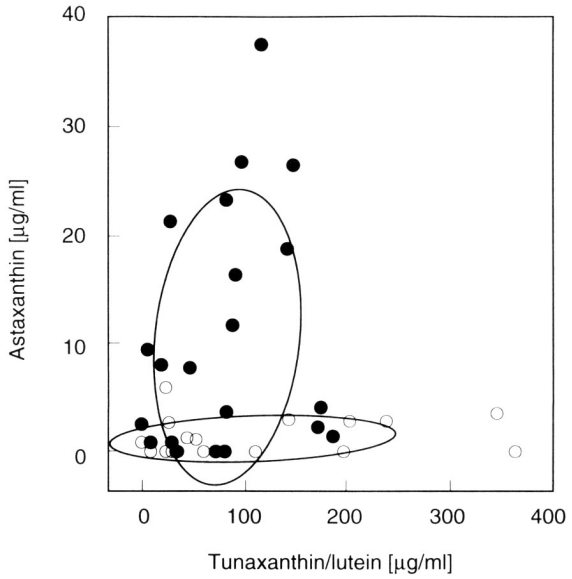


FIG. 5. Carotenoid content of sticklebacks that are described as yellow or intermediate red–yellow (open circles; color groups 1–3) and sticklebacks that are described as clearly more red than yellow (closed circles; color groups 4 and 5) in hexane–acetone (86:14) solution. The ellipses are superimposed on the two groups with a 50% confidence region of the data values. Disciminant analysis: $D = 2.80^*$ (astaxanthin) $- 0.09$ (tunaxanthin/lutein); $F = 7.30$, $df = 2$, $P = 0.002$.

considerably between individuals but did not appear to correlate within the fish. This indicates that the two groups of pigment we measured are physiologically independent of each other. Both correlate with the sticklebacks' coloration in a specific manner: tunaxanthin/lutein seems to cause a yellow or yellowish color, while astaxanthin correlated strongly with red color. Therefore, the red coloration of sticklebacks can be seen as a multiple ornament, where the type of carotenoid and the amount present in the fish vary independently. As a consequence, male sticklebacks could signal more detailed information to females than only their health and vigor (Hamilton and Zuk, 1982; Milinski and Bakker, 1990; Frischknecht, 1993), which is usually seen as a one-dimensional variable.

In our analyses of coloration we have neglected potential differences in the area of the skin that show conspicuous yellow or red color. Since we used the whole skin of the males for our carotenoid measurements, we do not know how much of the variance in pigment quantification that is left unexplained is connected to differences in pigment density over different areas of the skin. Accordingly, color intensity measured with the spectrometer (covering only an area of

about 2 mm^2 on the fish) does not seem to be strongly correlated to pigment content (see multivariate analyses). However, this should not have a great impact on the relative amount of the two groups of carotenoids found, since the sticklebacks' coloration in our sample appeared to be quite homogeneous, containing either yellow, red, or a mixture of these two colors.

The individual differences in pigment contents could reveal differences in food supply and food range. Since carotenoids cannot be synthesized by the fish (Simpson et al., 1981), they have to come from food sources. It is known that certain prey types promote stickleback coloration (T. Bakker, unpublished data), and different prey may contain different carotenoids. However, if the males of a given population do not differ greatly in their diet, the individual color differences could reveal the needs of male physiology at the time of mate choice. Males during the breeding season may be forced to balance the needs of being attractive and fighting infection. This may necessitate reallocation of carotenoids because carotenoids are important biological agents in different organs, especially for immune functions (Bendich, 1989, 1991; Bendich and Olson, 1989; West et al., 1991; Krinsky, 1993; Christiansen et al., 1995). This may be the reason why many sexual signals are based on carotenoids (e.g., Gray, 1996). By choosing colorful males, females get, on average, males with a lower parasite burden because the needs of the immune system correlate with it (Lozano, 1994). Accordingly, several studies showed that carotenoid-dependent coloring got weaker with infection in sticklebacks (Milinski and Bakker, 1990), red jungle fowl (Zuk et al., 1990), guppies (Houde and Torio, 1992), and Arctic char (Skarstein and Folstad, 1996). In Milinski and Bakker's (1990) experimental infection of male sticklebacks with the ectoparasite *Ichtyophthirius multifiliis*, the coloration not only paled after infection, but males who were more intensely colored at the time of infection developed a weaker infection (Milinski and Bakker, 1991). This again suggests that carotenoids can be recruited from the skin if they are necessary for immune defense.

We propose here an extension of Lozano's (1994) hypothesis on the connection between parasites, carotenoid-based signals, and sexual selection. Since carotenoids can differ in their chemical structure and their biological function (Bendich and Olson, 1989; Krinsky, 1993), different carotenoids are likely to differ in their impact on immune function. It is therefore possible that the amounts of different carotenoids an animal uses to fight its current parasite infections differ according to the kind of infection. The absolute and relative amounts of all carotenoids that constitute multiple coloration could then reveal detailed information about the current needs of the immune system and, therefore, about the kind and the intensity of a current infection. This may be the case, because the males are likely to redistribute their metabolites in an adaptive manner, taking into account the conflicting needs of being attractive versus fighting pathogens. The resulting detailed information in the breeding coloration could be

valuable for females and, consequently, may have a strong influence on their mate choice. A female that takes such detailed information into account could choose males with complementary disease resistances (Wedekind, 1992, 1994), while females that simply use a one-dimensional signal may only be able to choose males that are generally in good health and vigor (Hamilton and Zuk, 1982). We found here that the red coloration of sticklebacks has the potential to reveal detailed information. However, whether the two groups of carotenoids found here differ in their impact on the immune system and whether this information is used by females still needs to be tested.

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ANTIFEEDANTS TO ARMYWORMS, *Spodoptera litura* AND *Pseudaletia separata*, FROM BITTER GOURD LEAVES, *Momordica charantia*

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Abstract—Bitter gourd, *Momordica charantia*, was less palatable to two species of armyworms, *Spodoptera litura* and *Pseudaletia separata*, than two other cucurbitaceous plants. A methanol extract of *M. charantia* leaves inhibited feeding of the armyworm larvae. The two most active fractions obtained by silica gel chromatography were purified by HPLC. Momordicine II, a triterpene monoglucoside, was identified as an antifeedant compound from the more active of these fractions. The second active fraction led to the isolation of a new triterpene diglucoside. Fresh leaves of *M. charantia* contained ca. 0.3% of momordicine II. Momordicine II showed significant antifeedant effects on *P. separata* at concentrations of 0.02, 0.1, and 0.5% in artificial diets. Momordicine II caused a significant feeding reduction in *S. litura* only at the highest concentration (0.5%) tested. The difference in feeding response of the two armyworms to momordicine II may be related to the diversity in their host range.

Key Words—Bitter gourd, *Momordica charantia*, antifeedant, armyworm, *Spodoptera litura*, *Pseudaletia separata*, momordicine, triterpene glycoside, Cucurbitaceae.

INTRODUCTION

Bitter gourd, *Momordica charantia* L., is widely cultivated in tropical Africa and Asia as a vegetable crop. The leaves of *M. charantia* are less attacked by

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insects compared with those of other cucurbitaceous crops. Repellent and/or deterrent chemicals may play a major role in the unsuitability of nonhost plants as food for insects. The elucidation of these chemicals is important not only for understanding the ecological aspect of insect–plant relationships, but also for their potential in pest control.

The armyworm, *Spodoptera litura*, a well-known polyphagous insect, often has been used to evaluate antifeedants in plants (Wada and Munakata, 1968). A closely related armyworm, the paddy armyworm, *Pseudaletia separata*, feeds on mostly graminaceous plants and some other families. Its gregarious larvae show tolerances to relatively unpalatable food (Iwao, 1962).

In the present paper, we describe the feeding response of two polyphagous armyworms to three cucurbitaceous plants, including *M. charantia*, and also examined whether chemicals in *M. charantia* might play a major role in the rejection of feeding. We also report the isolation and identification of antifeedants from *M. charantia* leaves that are effective against *Spodoptera litura* and *Pseudaletia separata*.

METHODS AND MATERIALS

Insects. Spodoptera litura larvae were reared on an artificial diet (Wakamura, 1988) with soybean used instead of kidney bean. *Pseudaletia separata* larvae were reared on the Insecta LF artificial diet, (Nihon Nousan Kogyo Co.). Both insects were reared under gregarious conditions at 25°C and a 16L:8D photoperiod. Third or fourth-instars within a half day after ecdysis were used in bioassays.

Plants. Fresh leaves of cucumber *Cucumis sativus* L., pumpkin *Cucubita moschata* Duch., and bitter gourd *Momordica charantia* L. were obtained for bioassay from the plants grown in a greenhouse at our institute. *M. charantia* leaves were immediately freeze-dried for extraction.

Feeding Response of S. litura *and* P. separata *to Three Cucurbitaceous Plants.* Fresh leaves of cucumber, pumpkin, and bitter gourd were cut into 1.5-cm-diameter leaf discs with a cork borer. Two bitter gourd discs paired with either two cucumber or two pumpkin leaf discs were put on moistened filter paper in a 9-cm plastic dish. Six third instars were released in the Petri dish for 4 hr to test feeding response. Each test was replicated three times.

Bioassay for Antifeedants from Leaf Extract. Each fraction (1 mg) was dissolved in methanol (100:1) and applied (10 μl $\times$ 2) uniformly to both surfaces of the 1-cm-diameter leaf discs of cucumber and air dried. Leaf discs treated with methanol were used as a control. One test and one control leaf disc were kept on moistened filter paper in a 9-cm Petri dish. Five third instars were released in it and kept there for 12 hr. Each test was replicated three times. The extent of feeding was evaluated by measuring the area of the leaf consumed.

Feeding Response of S. litura *and* P. separata *to Isolated Compounds.* Each of the test compounds was added to the artificial diets at the concentrations of 0.02, 0.1, 0.5, and 2.5% (w/w). The artificial diet (150 mg) was placed in plastic microtube (1.5 ml). A single third instar was released in a tube and allowed to feed for 12 hr. Each test was replicated six times. The amount of feeding was evaluated by counting the number of fecal pellets. The number of fecal pellets was converted to a percentage based on number of pellets from the control diet.

Fractionation of Antifeedant Extracts from M. charantia *Leaves.* The fractionation procedure is shown in Figure 1. Each fraction was assayed after every step. Each active fraction was further fractionated and assayed repeatedly.

Isolation and Identification of Antifeedant Compounds. The active fractions obtained by column chromatography were each purified by high-performance

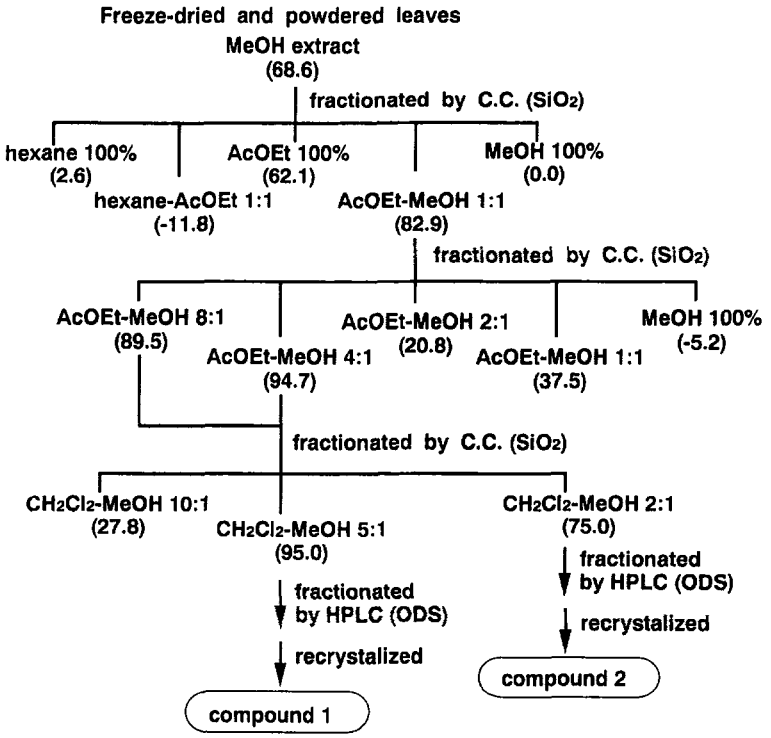

FIG. 1. Fractionation procedure of leaf extracts of *M. charantia* to isolate antifeedants. Values in parentheses are inhibition percentages compared with control. Inhibition (%) = 100 × (area consumed on control − area consumed on treated)/area consumed on control. One test and one control leaf disc were given to five third instars of *P. separata* in each test. C.C., column chromatography.

liquid chromatography (HPLC) (Shimadzu LC-10A system). HPLC was performed with a column of Cosmosil-5C_{18}-AR-II (Nacalai Tesque, Inc.) at 30°C. Compound **1** was eluted (2.8 ml/min) with 80% methanol, and compound **2** was eluted with 77% methanol. The UV detector was set at 210 nm. The crude compounds were recrystallized from MeOH–H_2O. NMR spectra were recorded with Jeol JNM-600 and Jeol JNM-400 spectrometers. Mass spectra (FAB) were obtained with a Jeol JMS-SX102A spectrometer.

β-D-glucosidase Hydrolysis. The mixture of 5 mg of antifeedant compound **1** and 1 mg (36 units) of β-glucosidase (Oriental Yeast Co.) in 0.4 ml of 0.02M acetate buffer, pH 5.0, was incubated at 37°C for 40 hr. The reaction mixture was extracted with diethyl ether twice. The ether layer was washed with water and dried over anhydrous sodium sulfate. After the solvent was evaporated, the extract was separated by column chromatography (hexane–ethyl acetate at 1:1, 1:2, and 1:4, and ethyl acetate 100%) to obtain 3 mg of compound **3.**

RESULTS

Feeding Response of S. litura *and* P. separata *to Three Cucurbitaceous Plants.* Among the three cucurbitaceous plants tested, *M. charantia* was less fed upon by both *S. litura* (Figure 2a) and *P. separata* (Figure 2b) larvae (*P*

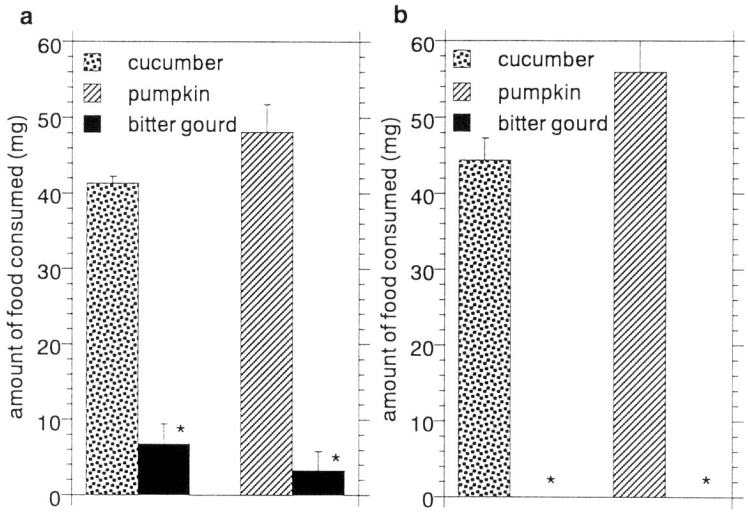

FIG. 2. Feeding response of *S. litura* and *P. separata* to three cucurbitaceous plants (two-choice test between bitter gourd and other cucurbitaceous plants). (a) *Spodoptera litura*, (b) *Pseudaletia separata*. *Significantly different at $P < 0.01$ (t test).

< 0.01, t test). In particular, no feeding was found in *M. charantia* by *P. separata*, although some biting marks were observed.

Bioassay for Antifeedant. Methanol was an effective solvent for extracting the antifeedants from *M. charantia* leaves, and the active components are polar. *P. separata*, which was more sensitive to *M. charantia* than *S. litura*, was used for the following bioassay. Each fraction was assayed after each fractionation procedure (Figure 1). Fractions that significantly reduced feeding were further fractionated to isolate the active components.

Identification of Antifeedant Compounds. Each compound isolated by the procedure shown in Figure 1 was identified by NMR, MS, and IR spectra.

The molecular ion peak (FAB) of compound **1** was observed at m/z 657 $(M + Na)^+$. Enzymatic hydrolysis of **1** with β-D-glucosidase gave compound **3.** These results and NMR spectral data suggested that **1** was a triterpene glucoside, and we identified it as momordicine II, the 23-*O*-β-glucopyranoside of 3,7,23-trihydroxycucurbita-5,24-dien-19-al (Figure 3). Spectral data (NMR, MS, and IR) of the isolated compound in this study were identical with those reported previously (Yasuda et al., 1984). NMR and MS (FAB) spectra of compound **3** obtained from the enzymatic hydrolysis of momordicine II was identified as momordicine I, the aglycon of momordicine II.

Compound **2** is present at about 0.08% of the fresh weight of leaves of *M. charantia*, and it was obtained as an amorphous powder. A molecular ion peak (FAB) of **2** observed at m/z 819 $(M + Na)^+$ was higher by 162 mass units than that of momordicine II, indicating the presence of one additional mole of the glycosyl group in **2.** The NMR spectra of **2** were very similar to those of **1** except that the appearance of additional anomeric proton (δ4.94) and β-glucopyranosyl group signals, and the signal at C-7 in **1** (δ65.75) moved ca. 6 ppm

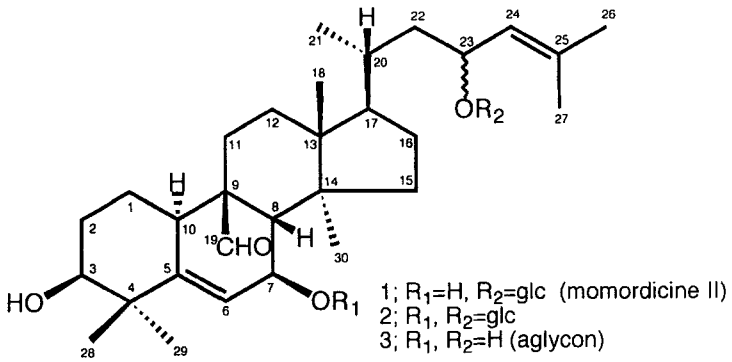

FIG. 3. Structure of momordicine II and its related compounds isolated from *M. charantia* leaves.

downfield (δ71.66). Thus, the additional glucopyranosyl moiety probably is linked to the C-7 hydroxyl group. This assumption was supported by the long range ^{1}H–^{13}C COSY spectrum of **2,** in which the anomeric proton at δ4.94 showed a long-range correlation with the carbon at δ71.66 (C-7). Additional information on the sugar linkage to the aglycon was obtained from NOESY spectrum. NOE was observed between the signals of δ4.94 (anomeric proton) and δ2.54 (H-8). The configuration of the glucosidic linkage of C-7 was determined as β based on the coupling constant (J = 8.0 Hz) of the anomeric proton. Signals of ^{1}H and ^{13}C spectra were all assigned by ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and long-range ^{1}H–^{13}C COSY spectra. From the above evidence, **2** was identified as the 7,23-di-*O*-β-glucopyranoside of 3,7,23-trihydroxycucurbita-5,24-dien-19-al (Figure 3). For comparison, ^{13}C spectral data of compounds **1, 2,** and **3** are shown in Table 1. From this table, it is evident that one glucopyranose is linked at C-23 in **1** and two glucopyranoses are linked in **2,** one each at C-7 and C-23. ^{1}H NMR spectral data of **2** are as follows (measured in C_5D_5N, 500 MHz): δ0.76 (3H, s, CH_3-30), 0.90 (3H, s, CH_3-18), 1.14 (3H, s, CH_3-29), 1.17 (3H, d, CH_3-21), 1.21 (1H, H-22), 1.45 (3H, s, CH_3-28), 1.45 (1H, H-15), 1.49 (1H, H-16), 1.54 (2H, H-15, 17), 1.57 (2H, H-11), 1.64 (1H, H-12), 1.68 (1H, H-1), 1.70 (3H, s, CH_3-27), 1.78 (3H, s, CH_3-28), 1.90 (1H, H-2), 1.94 (2H, H-1, 16), 2.04 (1H, H-2), 2.15 (1H, H-22), 2.16 (1H, H-20), 2.54 (1H, s, H-8), 2.64 (1H, H-10), 3.80 (1H, br. s, H-3), 3.93 (1H, m, 23-glc H-5′), 4.01 (1H, 7-glc H-5″), 4.02 (1H, 7-glc H-2″), 4.06 (1H, 23-glc H-2′), 4.26 (2H, 23-glc H-3′,4′), 4.29 (2H, 7-glc H-3″,4″), 4.37 (1H, 23-glc H-6′), 4.44 (1H, 7-glc H-6″), 4.50 (1H, 23-glc H-6′), 4.59 (1H, d, H-7), 4.64 (1H, 7-glc H-6″), 4.94 (1H, H-23), 4.94 (1H, d, 7-glc H-1″), 4.99 (1H, d, 23-glc H-1′), 5.65 (1H, d, H-24), 6.18 (1H, d, H-6), 10.49 (1H, s, H-19).

Feeding Response of S. litura *and* P. separata *to Isolated Compounds.* Compound **1** (momordicine II) had a significant ($P < 0.01$) antifeedant effect on *P. separata* at concentrations of 0.02, 0.1 and 0.5% in artificial diets (Figure 4). The extent of feeding reduction ranged from 45% to 60% at those concentrations. With *S. litura*, it caused significant ($P < 0.01$) feeding reduction at the highest two concentrations, 0.5 and 2.5%, respectively (Figure 5).

Compound **2** caused significant ($P < 0.05$) feeding reduction of 22% at the concentrations of 0.1 and 0.5% (Figure 6), but it caused no feeding reduction to *S. litura* at 2.5% concentration (data not shown).

DISCUSSION

We concentrated on one type of interaction mediated by chemicals concerned with feeding on a plant by polyphagous insects. Insects do not eat every

TABLE 1. ^{13}C NMR SPECTRAL DATA OF COMPOUNDS **1**, **2**, AND **3**[a]

C	1	2	3
1	21.79	21.90	21.81
2	29.96	29.83	29.93
3	75.69	75.58	75.72
4	41.81	41.95	41.82
5	145.74	145.68	147.73
6	124.33	124.28	122.36
7	65.75	71.66	65.76
8	50.62	45.01	50.64
9	50.71	50.41	50.72
10	36.87	36.69	36.92
11	22.71	22.64	22.77
12	29.64	29.50	29.64
13	45.92	45.85	45.97
14	48.30	48.17	48.30
15	34.96	34.86	34.93
16	27.86	27.80	28.10
17	51.30	51.34	51.26
18	14.98	14.99	15.01
19	207.90	207.42	207.83
20	32.71	32.75	32.93
21	19.45	19.64	19.14
22	43.80	43.94	45.48
23	75.33	75.48	65.19
24	129.18	129.19	131.95
25	132.28	132.15	130.85
26	18.29	18.31	18.27
27	26.27	26.29	26.24
28	25.88	25.88	25.79
29	27.35	27.34	27.37
30	18.24	18.18	18.10
23-glc 1′	104.24	104.43	
23-glc 2′	75.69	75.71	
23-glc 3′	78.98	78.97	
23-glc 4′	71.85	71.86	
23-glc 5′	78.35	78.34	
23-glc 6′	63.01	63.01	
7-glc 1″		101.69	
7-glc 2″		74.97	
7-glc 3″		78.79	
7-glc 4″		71.86	
7-glc 5″		78.90	
7-glc 6″		63.01	

[a]Measured in C_5D_5N solution.

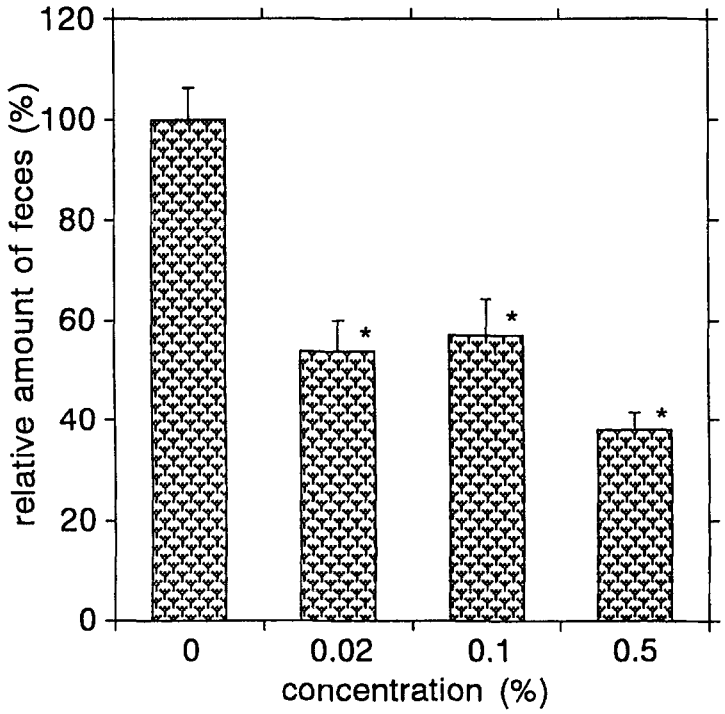

FIG. 4. Feeding response of *P. separata* to compound **1** (momordicine II) with four different concentrations. Artificial diet containing momordicine II was given to third instars. The number of fecal pellets was converted to a percentage based on fecal pellets from the control. *Significantly different at $P < 0.01$ (t test).

plant they encounter, and this is true even for species that eat a very wide range of food plants. Trichomes on plant surfaces are considered a defensive component of plants against herbivorous insects (Honda and Bowers, 1996) because of their physical or chemical properties. Although the plant surface of cucumber and pumpkin is covered with trichomes, unlike bitter gourd with a smooth surface, the former two plants were preferably fed upon by both armyworms in this study. Therefore, we suggest that such rejection of *M. charantia* leaves is primarily based on some chemicals not derived from trichomes. Our observation that the larvae bit the *M. charantia* leaves but never made sustained feeding suggests that some antifeedants in the *M. charantia* leaves caused the reduction of larval feeding rather than toxic or volatile repellent components. An antifeedant can be defined as a chemical that inhibits feeding but does not kill the

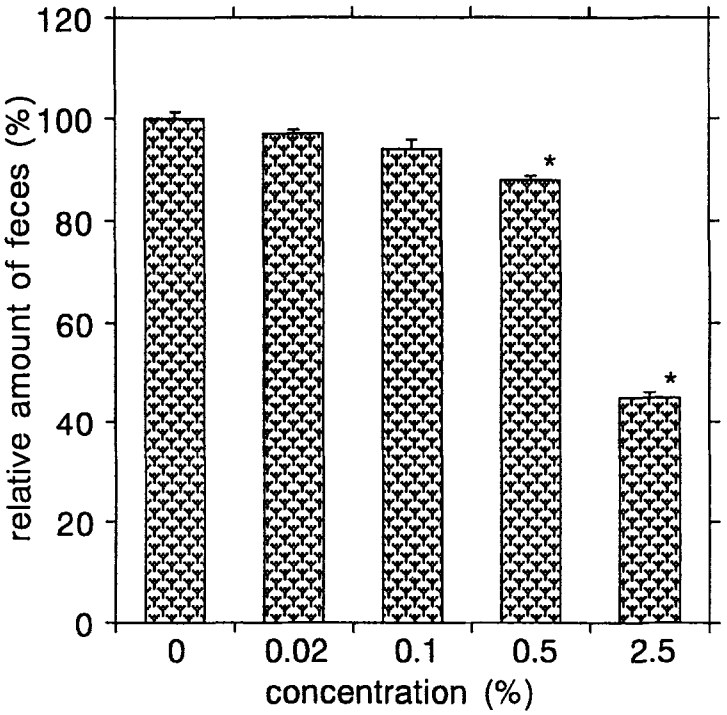

FIG. 5. Feeding response of *S. litura* to compound 1 (momordicine II) with five different concentrations. Artificial diet containing momordicine II was given to third instars. The number of fecal pellets was converted to a percentage based on fecal pellets from the control. *Significantly different at $P < 0.01$ (t test).

insect directly; the insect often may remain on the treated plant material and possibly may die of starvation.

Although cucurbitaceous plants contain secondary components such as cucurbitacins that protect against some insects (Ananthakrishnan and Raman, 1993), both armyworms in this study were able to accept cucumber and pumpkin leaves. On the other hand, *M. charantia* was not accepted by them. Thus, components contained only in *M. charantia* leaves caused a difference in feeding response of armyworm larvae between *M. charantia* and other two cucurbitaceous plants.

P. separata is more sensitive to momordicine II (**1**) than *S. litura*. The difference in feeding response to *M. charantia* leaves of the two armyworms can be explained by their different sensitivity to momordicine II. Although the

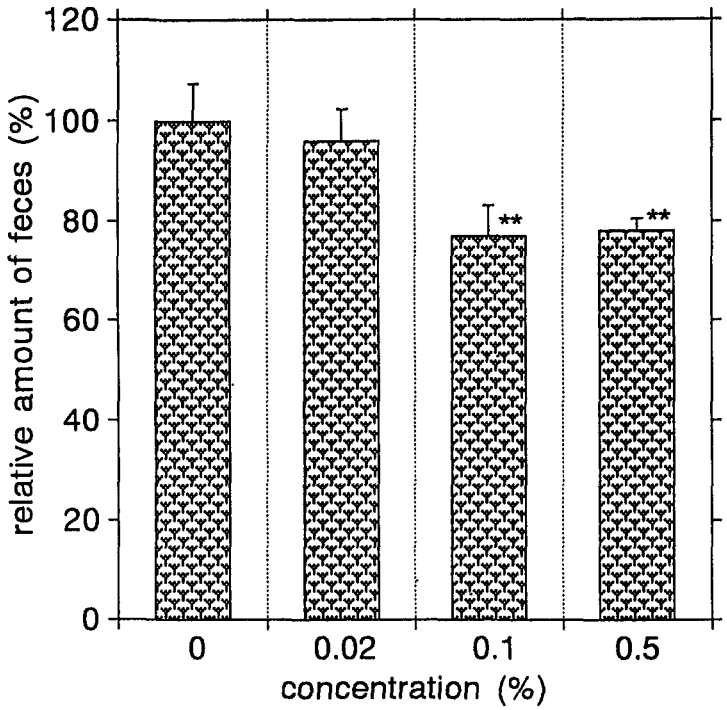

FIG. 6. Feeding response of *P. separata* to compound **2** with four different concentrations. Artificial diet containing compound **2** was given to third instars. The number of fecal pellets was converted to a percentage based on the fecal pellets from the control. **Significantly different at $P < 0.05$ (t test).

larvae of *P. separata* used in this study were gregarious-phase larvae that have been shown to have higher tolerances to some unpalatable foods than solitary-phase larvae (Iwao, 1962), their feeding on *M. charantia* and on the momordicines was strongly inhibited. The lower sensitivity of *S. litura* to Momordicine II correlates with its strong polyphagy. The fresh leaves of *M. charantia* containing ca. 0.3% of momordicine II completely inhibited the feeding of *P. separata*, while 0.5% of isolated momordicine II in the artificial diet was required to cause 60% inhibition of feeding by *P. separata*. This suggests that some other compounds also may be involved in antifeedant activity of the fresh leaves. Six compounds have been isolated from the leaves of *M. charantia*, and all of those compounds were cucurbitane triterpenoids, including momordicine I and II (Yasuda et al., 1984, Fatope et al., 1990).

Kumar et al. (1979) found that *M. charantia* seed oil emulsion had an antifeeding effect and insecticidal properties against the larvae of mustard sawfly,

Athalis proxima Klug. Chandravadana and Pal (1983) also reported that the cotyledons and leaves of *M. charantia* were unacceptable to red pumpkin beetles, *Aulacophr foveicollis* Lucas. Momordicine II was identified as a major antifeedant in the leaves (Chandravadana, 1987). The present study demonstrated that momordicine II and its newly identified related compound had antifeedant activities against two lepidopteran species with different polyphagy levels.

The fresh leaves of *M. charantia* contain ca. 0.1% compound **2**, and its antifeedant activity is lower than that of momordicine II. Momordicine I (**3**, the aglycon of momordicine II) did not show significant antifeedant activity even at the 0.5% level (data not shown). Among these three compounds (momordicine I, II, and compound **2**), the difference in antifeedant activities may be related to a difference in their glucopyranose moiety that contributes to the polarity. The moderate polarity of the compounds might be one of the important factors contributing to the activity.

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VARIATION OF PIPERIDINE ALKALOIDS IN PONDEROSA (*Pinus ponderosa*) AND LODGEPOLE PINE (*P. contorta*) FOLIAGE FROM CENTRAL OREGON

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Abstract—We quantified 2,6-disubstituted piperidine alkaloids in *Pinus ponderosa* and *P. contorta* needles from three forest sites in April, June, August, and December. Alkaloids were detected on at least one date in 71% of the ponderosa pine and in 29% of the lodgepole pine trees sampled. Pinidine was the major alkaloid constituent of ponderosa pine, while euphococcinine was the predominant compound in lodgepole pine. For ponderosa pine, total alkaloid concentrations were very low at two sites on all dates. At the third site, concentrations were variable but significantly higher on all dates. Total alkaloid concentrations in previous-year foliage from this site were highest in April, then significantly lower from June through December. Current-year foliage collected in August and December had significantly higher alkaloid concentrations than previous-year foliage on the same dates. Variation in foliar nitrogen concentrations accounted for some of the alkaloid variation in current-year foliage sampled in August.

Key Words—Piperidine alkaloids, pinidine, euphococcinine, foliar chemistry, nitrogen, *Pinus ponderosa*, *Pinus contorta*.

INTRODUCTION

Surveys of piperidine alkaloids in conifers (*Pinus* spp. and *Picea* spp.) have indicated a high degree of qualitative and quantitative variability among species, and among tissues within species (Stermitz et al., 1994; Tallent et al., 1955; Tawara, 1994; Tawara et al., 1993; Todd et al., 1995). Quantitative analyses of young, greenhouse-grown seedlings have described alkaloid variation and

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metamorphosis with age (Tawara et al., 1995; Todd, 1994). For *Pinus ponderosa*, Dougl. ex Laws, pinidine [*cis*-2-methyl-6-(2-propenyl)piperidine] and euphococcinine (1-methyl-9-*nor*-3-granatanone) appear to be final products of biosynthetic pathways involving 1,2-dehydropinidinone, pinidinone, 1,2-dehydropinidinol, and pinidinol as intermediates (Tawara et al., 1993, 1995) (Figure 1).

Our primary objective was to assess the variability of these alkaloids in foliage of trees growing in different forest conditions. We quantified piperidine alkaloids in the two dominant pine species of central Oregon (*Pinus ponderosa* and *P. contorta*, Dougl. ex Loud.) at three sites with differing environmental and silvicultural regimes. Seasonal variation was evaluated by sampling in April, June, August, and December. The August and December collections included current-year needles, in addition to previous-year needles, to assess variation

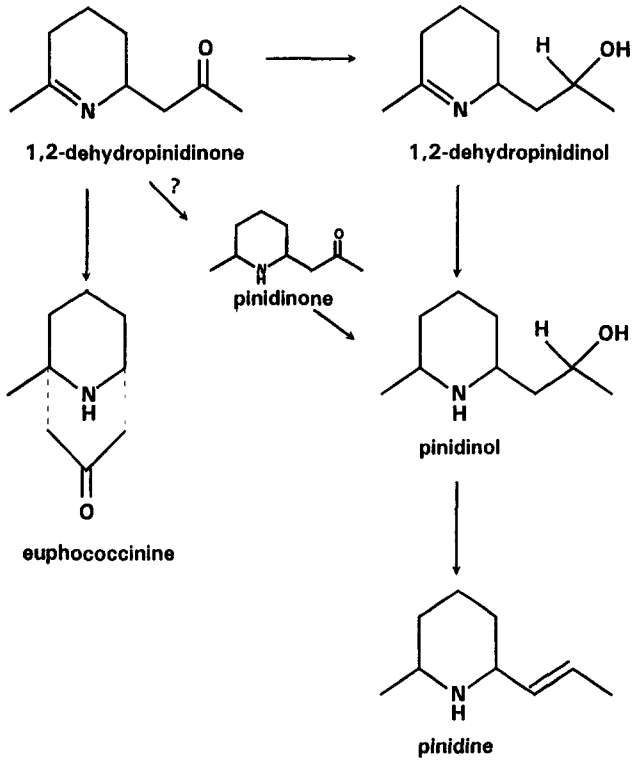

FIG. 1. Hypothetical biosynthetic pathways for piperidine alkaloids found in *Pinus* spp. (Reprinted with permission from *The Journal of Organic Chemistry*, 1993, 58:4816. Copyright 1993 American Chemical Society).

with tissue age. Soil nitrogen availability and total nitrogen concentrations in the foliage collected in August were measured for comparison with alkaloid concentrations. Additional growth-related parameters were measured on the sample trees to characterize differences among the sites and to identify potential correlates to alkaloid production. *Pinus ponderosa* foliage was sampled repeatedly in a 24-hr period at one site to verify that there was no sampling bias due to diurnal variation.

METHODS AND MATERIALS

Site Descriptions. Sites were chosen to represent three distinct plant community types in the pumice region of the Deschutes National Forest, Oregon. Site A is a *P. ponderosa* plantation, approximately 50 years old, unthinned with 28 m^2/ha basal area, located at 4863 km N, 632 km E (UTM zone 10), and 1370 m elevation. *Pinus contorta* is a very minor component of the overstory, and *Purshia tridentata* (Pursh) DC dominates the understory. Site B is a mixed stand of *P. ponderosa* and *P. contorta* at 4856.5 km N, 634 km E, and 1495 m elevation. This site was naturally regenerated after logging 60 years ago, thinned in 1975 and 1992, and burned by wildfire in August 1995, leaving 14.7 m^2/ha basal area and a sparse understory of *P. tridentata* and *Arctostaphylos patula* Greene. Site C is a *P. contorta*-dominated pumice flat with scattered mature *P. ponderosa* at 4844 km N, 610 km E, and 1310 m elevation. It was heavily thinned and pile-burned in 1990 to encourage *P. ponderosa* regeneration. Site C supported 4.8 m^2/ha basal area and a predominantly graminoid understory in the year it was sampled. Annual precipitation (38–43 cm) and site productivity (approx. 3.75 m^3/ha/yr) were equivalent for sites B and C, and slightly lower for site A (38 cm and 3.0 m^3/ha/yr, respectively).

Foliar Sampling. Eight *P. ponderosa* and eight *P. contorta* were randomly selected at each site, usually within a 50-m radius of an arbitrary plot center. Selected trees fit the following criteria: diameter >10 cm; mid-crown within 8.5 m of the ground; and minimal bark damage, beetle attack, dwarf mistletoe, or fire scorch. All three sites were sampled on four dates in 1996: April 25, June 21, August 16, and December 13 (8-, 8- and 16-week intervals), except for site B, which was inaccessible in December. On each date, site A was sampled between 08:00 and 10:30 hr, site B between 10:45 and 13:00 hr, and site C between 13:15 and 15:30 hr. Each tree was sampled within the middle third of the live crown. Two branches from the north aspect and two from the south were clipped with a pole pruner and stored separately (by aspect) in zip-lock bags. The samples were kept on Dry Ice in the field, and at $-36°C$ in the lab until analysis (26–67 days). In April and June, only previous-year needles were sampled; in August and December, both previous-year and current-year needles were sampled.

Diurnal Sampling. Four *P. ponderosa* trees not used for seasonal foliar sampling were selected at site C. All four trees were about 4.5 m tall with nearly full crowns. Four branches in the middle third of the crown on the NNE aspect and four on the SSW aspect were flagged. Two fascicles of needles from each flagged branch were sampled at 20:00 hr on June 27, and at 08:00, 12:00, and 16:00 hr on June 28. For each tree, needles were composited by aspect, and stored in zip-lock bags on Dry Ice in the field and at −36°C for several days in the lab. Skies were clear during the sample period and ambient temperature ranged from 10°C to 20.5°C.

Extraction of Foliage. Our extraction procedure was adapted from Wink et al. (1995), which facilitated large numbers of samples. Preliminary extractions via this procedure and the method of Tawara et al. (1993) provided comparable yields. Samples were processed in randomized sets of eight to minimize systematic error. In a cooler, needles were snipped from the fascicles, diced to <1 cm, and stored in a covered dish less than 30 min before weighing. In the lab, approximately 2 g of needles were accurately weighed into a centrifuge tube, and another 1 g was weighed into a vial for dry weight after 16 hr at 104°C. The 2-g sample was homogenized 30–45 sec in 10 ml of 0.5 M aqueous (aq.), HCl. The homogenizer was rinsed with another 10 ml aq. HCl, and the solutions combined. The tubes were laid on an orbital shaker for 60 min at 100 rpm, then centrifuged for 5 min at 2500 rpm. The supernatant was decanted, and adjusted to pH 11–13 with 6 M NaOH. This solution was loaded on an Extrelut QE prep column (20 ml capacity; EM Separations Technology) and allowed to adsorb for 3–5 min. The column was rinsed twice with 18 ml CH_2Cl_2 and the eluent evaporated at room temperature to <1 ml. The evaporated extract then was transferred to a 1-ml volumetric flask and stored overnight at 3°C. The extract was reduced under a gentle airstream to <0.5 ml before adding 0.5 ml internal standard solution and diluting to volume. The internal standard solution contained 2-ethylpiperidine (98%; ACROS Organics) in CH_2Cl_2.

GC-MS Analysis of Foliage Extracts. An HP5890 gas chromatograph with a J&W Scientific DB-1 capillary column (30 m, × 0.25 mm ID, 25-μm film thickness), an HP5970 mass selective detector, and an HP59970 GC-MS workstation were used for peak identification and quantitation. GC conditions were as follows: injector and transfer line 250°C; initial oven temperature at 85°C programmed at 5°C/min to 135°C; and then 20°C/min to 235°C. A calibration curve for the internal standard versus dihydropinidine was used to calculate response factors as a function of the peak areas of the alkaloids in each sample.

Nitrogen Analyses. The remainder of the diced foliage from the August collection was combined for each *P. ponderosa* tree by equal weights of tissues from N and S aspects, and stored at −36°C. The samples were oven dried at 60°C for 24 hr, ground to pass a 40-mesh screen, and then dried again at 60°C

for 16 hr. Total percentage nitrogen in the foliage was quantified by the Stable Isotope Research Unit, Department of Soil Science, Oregon State University (OSU) in a Dumas combustion apparatus coupled with an isotope ratio GC-MS.

Mineral soil from around each of the 24 *P. ponderosa* trees at sites A–C was sampled to a depth of 15 cm with a sand auger 26 days after the August foliage collection. Two cores from the N aspect and two cores from the S aspect, at 1 m and 2 m from the stem, were combined in ziplock bags, and stored with crushed ice in the field. In the lab, they were stored at 6°C for two days, then homogenized and sieved to <2 mm in size. A seven-day waterlogged incubation of the soil [after Bundy and Meisinger (1994), method 41-2.2.1.2.2] was conducted to provide an index of available N at each site. Soil extracts were analyzed for NH_4 and NO_3 by the Plant & Soil Analytical Lab, Department of Forest Science, OSU, with an Alpchem-RFA 300 rapid flow colorimetric analyzer.

Individual Tree Measurements. Tree diameters were measured at 1.4 m from the ground. The amount of live crown is expressed as a percentage of tree height. The amount of basal area surrounding each sample tree, measured with a 10-factor prism, was taken as an index of overstory competition. Lengths of previous-year branch growth and of previous- and current-year needles were measured on the samples collected in August. Branch lengths are averages of two north and two south aspect branches; needle lengths are averages of four north and four south aspect needles.

Statistical Analyses. No statistical analyses were done for *P. contorta* because of insufficient nonzero data. The following analyses refer to data for *P. ponderosa*. Total alkaloid concentrations are additive quantities of pinidine, euphococcinine, pinidinol, dehydropinidinol, and/or dehydropinidinone expressed as micrograms per gram dry weight. Alkaloid concentrations for N and S aspects were averaged by tree after paired t tests indicated no consistent differences between aspects (Proc MEANS; SAS Institute, Inc., 1996). The effects of site and season on total alkaloid concentrations were tested with a mixed linear model including sampling dates as repeated measures (Proc MIXED; SAS Institute, Inc., 1996). The effect of needle age (current-year vs. previous-year foliage) was tested with August and December data for site C with paired t tests. Foliar N and available N in the soil were compared among sites by analysis of variance, and also tested as covariates for total alkaloid concentration by general linear models that incorporated site effects (Proc GLM; SAS Institute, Inc., 1989). Diurnal effects were tested as repeated measures in SAS Proc MIXED. Responses, i.e., total alkaloid concentrations, were transformed to natural logarithms for homogeneity of variance. Comparisons between means were evaluated with Fisher's protected LSD at $\alpha = 0.05$. The means ($\bar{X}$) reported are back-transformed LS means.

RESULTS

Piperidine alkaloids were detected in 17 of 24 *P. ponderosa* trees (Figure 2), and in 7 of 24 *P. contorta* trees on at least one sampling date. Trees with alkaloids in April usually had alkaloids in June, August, and December. In *P. ponderosa*, pinidine generally constituted 100% of the total alkaloid concentrations, while euphococcinine never exceeded 8%. Intermediate compounds (1,2-dehydropinidinone, 1,2-dehydropinidinol, and/or pinidinol) were detected only at site C in previous-year foliage of one *P. ponderosa* in April, three in June, and two in August; they were detected in current-year foliage in three trees in August. Quantities of intermediates typically were very low, but several trees had 10–35% of total alkaloids as 1,2-dehydropinidinol. Of the *P. contorta*, one tree had distinctly higher alkaloid concentrations, entirely in the form of euphococcinine. Alkaloid profiles for the remaining trees varied considerably, with two trees having small quantities of pinidine. Pinidinone was not detected in either species. None of the intermediate compounds were detected in any trees in December.

Total alkaloid concentrations (dry weight basis) in previous-year foliage of *P. ponderosa* varied significantly, depending on the site and the time of year

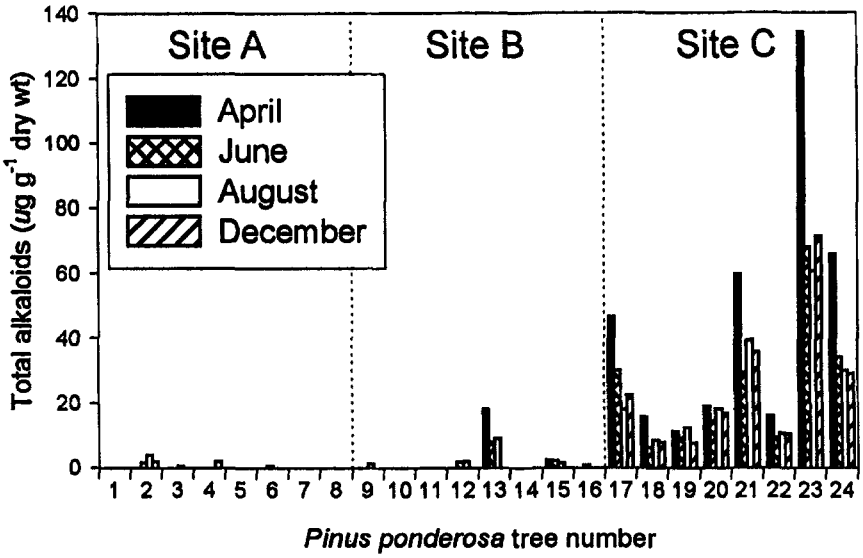

FIG. 2. Total alkaloid concentrations in previous-year foliage of 24 *P. ponderosa* trees from three sites in central Oregon. Mid-crown foliage was collected in April, June, August, and December, excepting site B in December. Bars represent averages for samples from north and south aspects.

($P = 0.010$). At sites A and B, foliar alkaloids were practically nonexistent throughout the year, whereas all trees at site C had piperidine alkaloids in their foliage on all dates (Figure 2). Mean alkaloid concentrations in foliage at site C were higher than at sites A and B on each sampling date (all site A and B $\bar{X} < 1$ μg/g dry wt; all $P < 0.001$). Mean alkaloid concentrations at sites A and B did not differ significantly for any sampling date. Total alkaloids in previous-year foliage at site C decreased from April to June ($P = 0.002$), then remained unchanged from June through August and December (Figure 3). If total alkaloid concentration is expressed on a fresh weight basis, distinctions among sites and dates remain the same. Total alkaloid concentrations of *P. contorta* foliage were <6 μg/g dry wt at all sites and dates, except for one tree at site C with 23–91 μg/g euphococcinine (highest in August).

For logistical reasons, site A was sampled in the morning and site C in the afternoon of each sampling date. To determine whether diurnal variation could explain why trees at site A always had very low alkaloid concentrations (<5 μg/g dry wt) and site C always had much higher alkaloid levels (Figure 2), we sampled four additional *P. ponderosa* trees at site C within a 24-hr period in June. Total alkaloid concentrations in these four trees differed quantitatively, but the variation associated with time of day was insignificant ($P = 0.999$; Figure 4). None of these trees had alkaloid levels at any time of the day that were as low as those observed at site A. Although this analysis does not rule out the possibility that diurnal variation may occur in *Pinus* spp., it does indicate that the large differences in alkaloid concentrations between our study sites were not caused by diurnal variation.

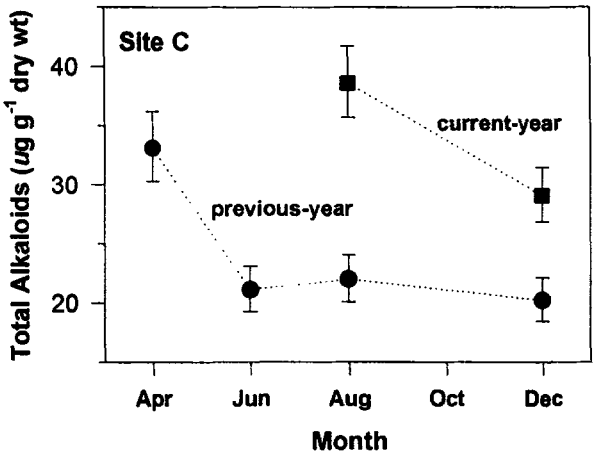

FIG. 3. Total alkaloid concentrations of previous- and current-year foliage from site C. Bars represent standard errors for differences between months, $N = 8$.

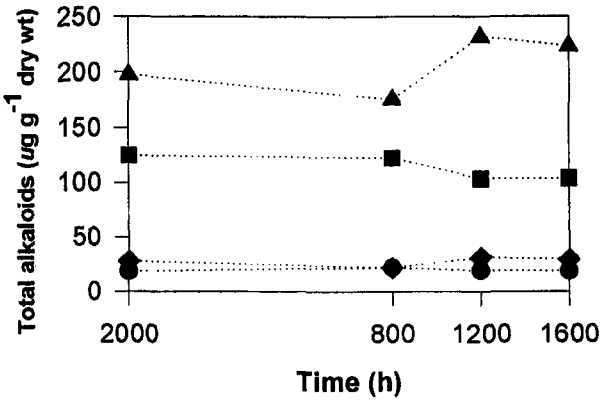

FIG. 4. Diurnal variation in foliar alkaloid concentration of four *P. ponderosa* trees at site C during a 24-hr period in June. Data points are averages for mid-crown samples from north and south aspects.

Total alkaloid concentrations in current-year foliage of *P. ponderosa* at site C decreased from August to December ($P < 0.001$; Figure 3), but alkaloid concentrations in current-year foliage remained higher than previous-year foliage on both dates (August, $P = 0.007$; December, $P = 0.034$). Current-year foliage at sites A and B had alkaloid concentrations of nearly zero in August (and December for site A). *Pinus contorta* was not sampled in December, but, of the five trees with alkaloids in August, all had lower concentrations in current-year foliage than in previous-year foliage.

Total N concentrations in current-year and previous-year *P. ponderosa* foliage collected in August were lowest at site A and highest at site C (Figure 5a). The difference in foliar N between site C and the other sites was stronger

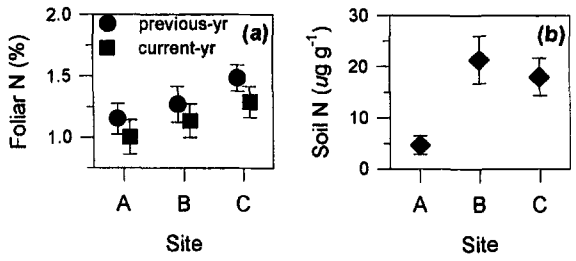

FIG. 5. Means by site for (a) total percent nitrogen in foliage collected in August, and (b) available nitrogen in soil. Bars represent 95% confidence intervals for $N = 8$ sample trees.

for previous-year ($\bar{X}_C:\bar{X}_B$, $P = 0.011$; $\bar{X}_C:\bar{X}_A$, $P < 0.001$; $\bar{X}_B:\bar{X}_A$, $P = 0.144$) than for current-year foliage ($\bar{X}_C:\bar{X}_B$, $P = 0.073$; $\bar{X}_C:\bar{X}_A$, $P = 0.002$; $\bar{X}_B:\bar{X}_A$, $P = 0.107$; Figure 5a). Foliar N was a significant covariate for alkaloid concentrations in current-year foliage across all sites ($P = 0.028$) and in previous-year foliage at site B ($P = 0.007$). After accounting for foliar N effects, alkaloid concentrations in current- and previous-year foliage were still dependent on other factors associated with the different sites ($P < 0.001$ and $P = 0.013$, respectively). Available N in the soil at site A never exceeded 7 μg/g, while sites B and C had higher but comparable levels of available N ($\bar{X}_C:\bar{X}_B$, $P = 0.137$; $\bar{X}_A:\bar{X}_C$, $P < 0.001$; Figure 5b). Available N in the soil was not a significant covariate for total alkaloids in current- or previous-year foliage.

Overall, sample tree sizes were similar across the three sites. Site C had the widest range of tree diameters (Figure 6a) and tree heights (Figure 6b), but

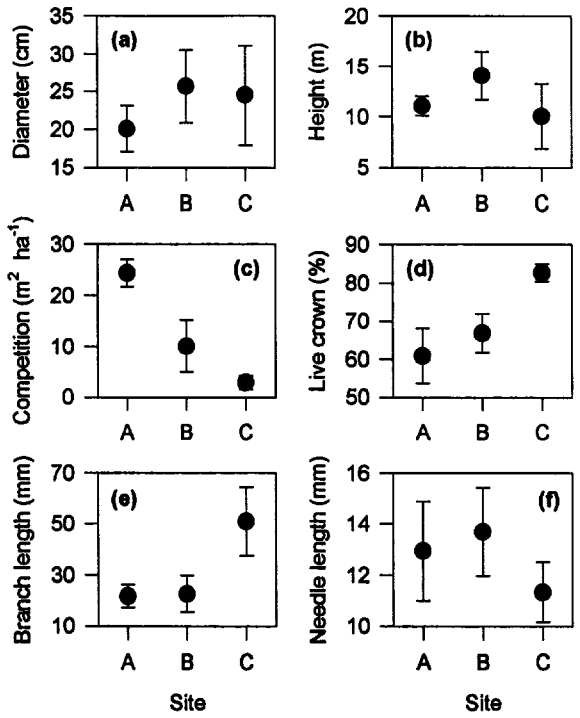

FIG. 6. Means by site for sample tree (a) diameter at 1.4 m, (b) height, (c) competition from neighboring trees, (d) amount of live crown as a percentage of tree height, (e) length of previous-year branch growth, and (f) current-year needle length. Bars represent 95% confidence intervals for $N = 8$ sample trees.

none of the means were distinctly different, so ontogenetic effects were unlikely to confound our comparisons. Overstory competition surrounding the sample trees was significantly lower at site C (Figure 6c), and, correspondingly, live crown percentages were distinctly higher at this site (Figure 6d). Elongation of branches supporting previous-year needles was greater at site C (Figure 6e), but lengths of previous-year (data not shown) and current-year needles (Figure 6f) were somewhat shorter at site C.

DISCUSSION

Species Differences. Piperidine alkaloid concentrations in foliage varied qualitatively and quantitatively between species. Pinidine, the most abundant alkaloid in *P. ponderosa*, was rarely detected in *P. contorta*, and euphococcinine, the most abundant alkaloid in *P. contorta*, was a minor constituent of *P. ponderosa*. These results are consistent with the different biosynthetic pathways proposed for *Pinus* spp. (Stermitz et al., 1994; Tawara et al., 1993, 1995) in which pinidine and euphococcinine are final products. *Pinus ponderosa* at site C apparently utilizes both pathways, but favors the pinidine pathway. Piperidine alkaloids have not been reported previously in *P. contorta*, perhaps because of their sporadic occurrence.

Variation with Season and Age of Needles. The decline in alkaloids in older *P. ponderosa* foliage at site C coincides with new needle elongation in central Oregon, which begins around June and continues through August. This decline could be caused by translocation of alkaloids in previous-year foliage to current-year foliage, or it could reflect breakdown processes or dilution effects associated with carbon deposition in the older foliage. Alkaloid levels tend to be higher in younger leaves (McKey, 1979), but Todd et al. (1995) measured higher concentrations of piperidine alkaloids in older needles of *Picea pungens* Engelm. in March.

Nitrogen Effects. Some of the variation in alkaloid concentrations was explained by differences in foliar N among the study sites. The site with the most alkaloids (site C) also had the highest foliar N levels, and the site with only trace amounts of alkaloids (site A) had substantially lower foliar N levels. Alkaloid and foliar N concentrations at site B were more similar to site A than to site C. Branch growth, percentage live crown, and lack of competition also corresponded closely to alkaloid concentrations, probably reflecting the influence of favorable nutrient status on alkaloid production. The relationship between alkaloid concentrations and foliar N was stronger for current-year foliage, probably because alkaloid concentrations in older foliage had more time to be influenced by translocation, breakdown, or developmental processes. Alkaloid production in lupines also is dependent on plant N content and tissue age (Johnson et al., 1987).

Available N in the soil was not a reliable indicator of foliar alkaloid content. Alkaloids and soil N were low at site A and relatively high at site C, but at site B, alkaloids were low and soil N was high. The recent fire history of site B probably explains the lack of correlation between alkaloids and soil N. Prescribed burning in central Oregon pine stands causes a temporary increase in availability of soil N in the year following a fire (Monleon et al., 1997). Foliar N may not be affected in the growing season following fire, and needle loss may reduce the overall N content of the crown (Landsberg et al., 1984). These factors probably limited alkaloid concentrations in both previous- and current-year needles at site B, even though N availability in the soil was high in September 1996.

Genetic Variation. Genetic differences in *P. ponderosa* may have contributed to the remaining variability in total alkaloids among sites. Trees at site A were planted about 50 years ago, probably from an off-site seed source, while trees at sites B and C were naturally regenerated (Bend-Ft. Rock R.D., Deschutes N.F., records on file). Trees at site A would be genetically most distinct, and this could explain the virtual absence of alkaloids. However, alkaloid concentrations at site B were more similar to site A than to site C, even though trees at sites B and C regenerated from local populations. Yet, site C is 27 km southwest and 185 m lower in elevation than site B, so genetic differences between the sites cannot be discounted. Substantial variability in quinolizidine alkaloids among sites have been attributed to genetic differences within a single lupine species (Wink and Carey, 1994).

Herbivory. Alkaloid concentrations in a variety of plants have been shown to increase or decrease in response to herbivory, depending on conditions such as degree of defoliation, and nutrient or water status of the plant (Brown and Trigo, 1995). Herbivory effects on alkaloids in the Pinaceae are unknown, but defoliation can cause a variety of physiological responses in conifers (Clancy et al., 1995) that could influence piperidine alkaloid production. All three of our study sites were defoliated to some extent in recent years by *Coloradia pandora* larvae, so the alkaloid concentrations we observed may have been influenced by this herbivory. *Coloradia pandora* feed on *P. ponderosa* and *P. lodgepole* until mid-June of alternate years. They finish their larval stage prior to current-year needle elongation in central Oregon, so defoliation directly impacts only older foliage. According to aerial survey classifications, site A was heavily defoliated in 1992 and lightly defoliated in 1994; site B was moderately defoliated in 1990 and 1992; and site C was lightly defoliated in 1994 and 1996 (USDA Forest Service, Forest Insects and Diseases, Region 6, Portland, Oregon). When samples were collected in June 1996, most trees at site C were lightly defoliated (we estimated about 10% of foliar biomass), whereas no defoliation was noted at sites A or B. Thus, any short-term, induced alkaloid response would be expected at site C. This site did have the highest alkaloid concentrations, yet total alkaloid concentrations in previous-year foliage at site

C declined from April to June while the larvae increased in size and defoliation accumulated. Apparently, light herbivory of these trees did not induce piperidine alkaloid production in older foliage. However, the relatively high alkaloid concentrations in current-year foliage at site C could represent translocation or de novo synthesis of alkaloids in younger, more valuable needles, in response to defoliation of the older needles. Pinaceae alkaloids are known to have antifeedant activity against eastern spruce budworm [*Choristoneura fumiferana* (Clem.)] (Schneider et al., 1991) and variegated cutworm [*Peridroma saucia* (Hubner)] (Stermitz et al., 1994).

Summary and Conclusion. This study demonstrated that foliar alkaloid concentrations in *P. ponderosa* can vary greatly from one locality to another, to the extent that piperidine alkaloids may be virtually absent throughout the year from foliage at a particular site. The age of foliage and time of year also affect alkaloids, with younger foliage having higher concentrations. The relative influence of genetics, abiotic environment, and herbivory remains to be determined. Clearly, assessments of piperidine alkaloids in conifers require robust sampling methodologies. Favorable nutrient status measured in terms of foliar nitrogen and growth correlates positively with alkaloid concentrations. Managing *P. ponderosa* at lower stand densities may favor higher alkaloid concentrations by increasing the amount of nutrients available to residual trees. This could be a desirable outcome if these potentially bioactive compounds (Schneider et al., 1991; Stermitz et al., 1994; Tawara et al., 1993) inhibit important herbivores or pathogens.

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DITERPENOID ALKALOID CONCENTRATION IN TALL LARKSPUR PLANTS DAMAGED BY LARKSPUR MIRID

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Abstract—Tall larkspur (*Delphinium barbeyi*) is a serious poisonous plant threat to cattle on mountain rangelands. The larkspur mirid [*Hopplomachus affiguratus*] has been proposed as a biological tool to damage tall larkspur in an effort to deter grazing by cattle and thus prevent poisoning. Preliminary data suggested that it may also reduce toxic alkaloid levels. The objective of this study was to determine if damage caused by the larkspur mirid would reduce toxic alkaloid concentration. Larkspur mirids were collected in the field in 1992 and placed on potted plants in the greenhouse. The resulting mirid-damaged leaves were lower in toxic alkaloids than leaves from uninfested plants. In the 1995 field study, toxic and total norditerpenoid alkaloid concentrations were measured in two larkspur populations having established mirid populations and in two newly infested larkspur populations. In the 1996 field study, three widely separated larkspur populations infested with mirids were sampled. Mirid-damaged leaves were lower in toxic alkaloids in both years, but there were no differences in flowering heads. However, only at Yampa, Colorado, did mirids reduce toxic alkaloids to levels that would not pose a threat to cattle. There was no difference in toxic or total alkaloid concentration between larkspur populations with long-term mirid infestations compared to newly infested plants. The plant-to-plant variability in alkaloid concentration was greater than differences due to mirids.

Key Words—Biological control, *Hopplomachus affiguratus*, Heteroptera, Miridae, cattle poisoning, *Delphinium barbeyi*, norditerpenoid alkaloids, methyllycaconitine.

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INTRODUCTION

Tall larkspur (*Delphinium barbeyi*) is a serious poisonous plant threat to cattle on mountain rangelands. The larkspur mirid [*Hopplomachus affiguratus* (Heteroptera: Miridae)] is a native insect that is host-specific to tall larkspur (Fitz, 1972; Uhler, 1895). It is a sucking insect that extracts cell solubles from immature, rapidly growing plant parts. The insects concentrate on flowering racemes, causing the buds to abort, and also damage the leaves, which first appear mottled, then desiccate and senesce. These are the larkspur plant parts normally preferred by cattle (Pfister et al., 1988a) and cattle will not graze mirid-damage larkspur plants (Ralphs et al., 1997a). Thus, the larkspur mirid has been proposed as a biological tool to damage tall larkspur in an effort to deter grazing by cattle and thus prevent poisoning.

Preliminary data indicate that toxic alkaloid concentration was lower in mirid-damaged larkspur plants than in undamaged plants (Ralphs unpublished data). Therefore, the larkspur mirid may have the potential to reduce larkspur toxicity, as well as its palatability. The objectives of this study were to determine the influence of mirid damage on toxic and total alkaloid concentration in tall larkspur and to determine if long-term mirid infestations suppressed alkaloid levels in larkspur populations compared to newly infested plants.

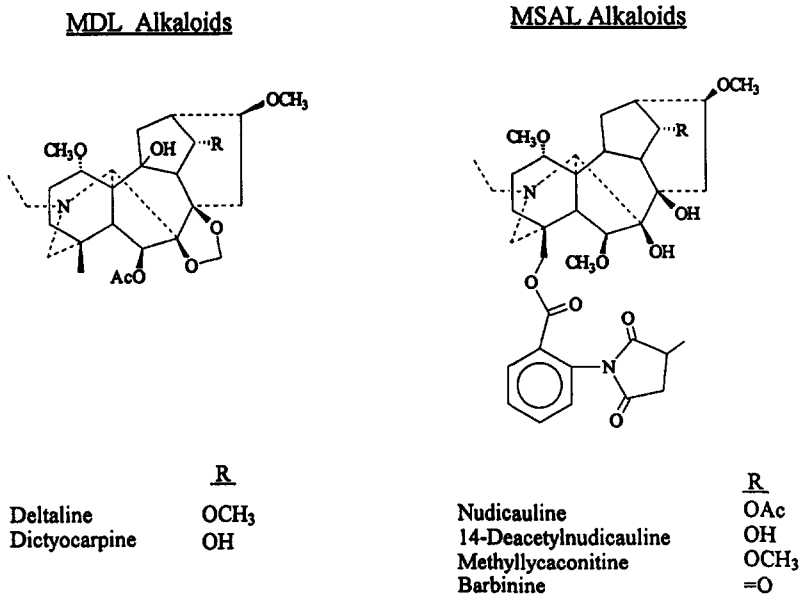
METHODS AND MATERIALS

Larkspur Alkaloids

The toxic compounds in larkspurs have been identified as norditerpenoid alkaloids (Figure 1). Alkaloids that contain the *N*-(methylsuccinimido) anthranilic ester group (referred to as MSAL alkaloids) are the most toxic (Manners et al., 1995), with methyllycaconitine (MLA) and 14-deacetylnudicauline (DAN) being the two most prominent toxic alkaloids in tall larkspur. In the preliminary greenhouse study, concentration of MLA, the dominant toxic alkaloid, was measured by high-pressure liquid chromatography (HPLC) following extraction in ethanol and chloroform (Manners and Pfister, 1993). The MSAL fraction (referred to as toxic alkaloids) and total alkaloid concentration in the 1995 and 1996 studies were analyzed by Fourier-transformed infrared spectroscopy (FTIR) (Gardner et al., 1997). The alkaloids were extracted in chloroform and 1% H_2SO_4, and IR spectra were collected with a Nicolet Magna 550 FT-IR spectrometer (Nicolet Instrument Corp., Madison, Wisconsin).

Preliminary Study

A dense population of the larkspur mirid exists in a tall larkspur patch near Ferron Reservoir, 45 km west of Ferron in east-central Utah. In 1992, a large



	R
Deltaline	OCH_3
Dictyocarpine	OH

	R
Nudicauline	OAc
14-Deacetylnudicauline	OH
Methyllycaconitine	OCH_3
Barbinine	=O

FIG. 1. Norditerpenoid alkaloids in tall larkspur. The two major classes of alkaloids are methylenedioxylycoctonine (MDL) and *N*-(methylsuccinimido)anthranoyllycoctonine (MSAL) alkaloids.

number of live nymphs and adult mirids were collected from the field and transferred to six potted larkspur plants in our greenhouse in Logan. After three weeks, leaves from these six plants were harvested, along with leaves from six control plants. All samples were dried in a forced air oven at 60°C, ground in a Wiley mill to pass through a 1-mm screen, and analyzed for MLA by HPLC.

1995 Study

The study was conducted on the Wasatch Plateau 45 km west of Ferron in central Utah. Four sites were selected in the subalpine zone (3000–3300 m elevation). The vegetation community consisted of scattered subalpine fir stands interspersed in the tall forb plant community dominated by tall larkspur, western cone flower (*Rudbeckia occidentalis*), sweet cicely (*Osmorhiza occidentalis*), and mountain brome (*Bromus carinatus*). Tall larkspur is a large robust native perennial forb. It has an average of 40 stems/plant (ranging up to 150 stems/plant) and grows to a height of 1 m in this area. It generally occurs in patches associated with snow drifts. The growing season is short at this elevation; growth begins as the snow melts in mid-June, and freezing temperatures curtail growth in early September.

The objective of this study was to compare the alkaloid concentration in mirid-damaged and undamaged parts of plants from larkspur populations where mirids have been abundant in the past, compared to larkspur populations in which the mirids were recently transplanted. Two sites were selected in each population type.

Existing Mirid Population. The first site was 3 km west of Ferron Reservoir. We first discovered the larkspur mirid in this area in 1989 in a 3-ha larkspur patch and have observed significant mirid damage each year. We estimated the mirid density at about 10,000 mirids per plant. There was an average of 90 mirids/leaf on the younger leaves on the upper half of the stem (6 leaves/stem), on about half the stems of each plant (20 stems/plant) at a given time. The mirids did not uniformly cover each plant. They were concentrated on the southeast quarter of the plant at the beginning of the season (perhaps due to the orientation of the morning sun), then spread throughout the plant as the season progressed. About half of each plant was damaged at the time the samples were harvested.

The second site was on 6-Mile Bench about 12 km north west of the first site. We transplanted the larkspur mirid to this location in 1993, and its population increased and spread throughout the 0.5-ha patch. Larkspur was in the early flower stage at Ferron Reservoir (0.9 m tall), and the early bud stage at 6-Mile (0.6 m tall), on August 8, 1995, when the samples were harvested.

There is a tremendous amount of variability in toxic alkaloid concentration among larkspur plants (Manners and Pfister, 1996). Therefore, samples were taken from a plant with both mirid-damaged and undamaged leaves and flowering heads. We randomly selected 10 larkspur plants at each location that had significant mirid damage. We selected about 15 flowering racemes (heads) and 50 leaves that were damaged, and the same number of heads and leaves without mirid damage from each plant. The respective samples were placed in paper bags, transported to our lab, and processed as described above and analyzed for total and toxic alkaloids by FTIR.

Transplant Population. Nymphs in the second to fourth instar were taken from the Ferron Reservoir population and transplanted at two uninfested larkspur patches in Duck Fork (4 km north) and 12-Mile Canyon (8 km west). Mirids were gently shaken from the larkspur plants into an insect sweep net, then were placed in large white garbage bags containing several larkspur stems to prevent bunching up. They were placed on the new plants within an hour. Ten robust plants were selected at each location, and each plant was divided in half using a nylon mesh screen (1-mm openings). Sections of screen (0.9 m wide and 0.6 m tall) were stapled to 1.2-m surveyor's stakes, which were driven into the ground splitting the foliage of each plant in half. The screens were oriented east to west, and the mirids were randomly placed on either the south or north side of the screen. From 500 to 1000 nymphs were placed on each plant.

The nymphs were transplanted on August 8, and the plant parts were harvested on August 25. Undamaged plants were in the full flower stage at Duck Fork and in the bud elongation stage at 12-Mile when harvested. Larkspur heads and leaves from the mirid-damaged and undamaged portions of the plants were harvested and processed, and alkaloid concentration was measured by FTIR. Some of the heavily damaged leaves on the mirid side of the screen were totally desiccated. These leaves were kept separate and analyzed as a third type of leaf in this study (undamaged, mirid-damaged, desiccated).

The extent of mirid damage was estimated on these recently infested plants. A visual estimate was made of the percentage of desiccated leaves and the percentage of leaf biomass damaged. The numbers of aborted heads and normal heads on each side of the screen were counted, and the percentage of aborted heads was calculated.

1996 Study

The objective of this study was to compare the toxic and total alkaloid concentration in mirid-damaged and undamaged plant parts in larkspur populations over a wide geographical area. Larkspur samples were collected from mirid-damaged larkspur patches on the Wasatch Plateau in central Utah 22 km east of Mayfield, on the Fishlake National Forest in south-central Utah 46 km east of Salina, and on the Routt National Forest 20 km west of Yampa on the western slope of Colorado.

The Mayfield site is in the same general area as the 1995 study, but on the west slope of the mountain. The Salina site was 50 km south at a similar elevation and plant community. The Yampa site was in an open park within the aspen vegetation zone at 2500 m elevation. Dominant species included tall larkspur, elderberry (*Sambucus racemosa*), saw groundsel (*Senecio serra*), and mountain brome (*Bromus carinatus*).

Mirid-damaged and undamaged leaves were taken from the same plant at Mayfield and Salina. Ten plants were randomly selected at each site. From 30 to 50 mirid-damaged and undamaged leaves were taken from the upper half of each plant at both sites, and 10–15 damaged and undamaged heads were taken from each plant at the Mayfield site. There were few undamaged heads at the Salina site; therefore only leaf samples were taken. At Yampa, infested plants were uniformly damaged (there were no undamaged plant parts on infested plants); therefore leaves and heads were harvested from six mirid-damaged and six undamaged larkspur plants. Alkaloids were measured by FTIR.

Nutrient Quality. Nutrient quality of mirid-damaged and undamaged leaves was also measured. Nitrogen was measured by an automated combustion method (Sweeney, 1989) that quantifies total N by thermal conductivity. Total N was multiplied by 6.25 to obtain crude protein (CP). Neutral detergent fiber (NDF)

was measured by the filter bag technique (Komarek et al., 1994) using standard NDF solution. NDF is a measure of cell walls, with its inverse being soluble cell contents. Sugar analysis was attempted, but results were inconclusive due to the large losses of soluble carbohydrate in the drying method.

Statistical Analysis of Data

The 1992 data from the preliminary greenhouse study were analyzed by one-way analysis of variance (ANOVA) with comparison of toxic alkaloid concentration in mirid-damaged and undamaged leaves. The two field studies were first analyzed singly, then combined and analyzed together in a large general linear model (GLM).

In the 1995 study, the concentration of toxic and total alkaloids in larkspur plant parts were compared between the existing mirid populations and transplanted populations by ANOVA. Differences between populations were tested by the site (within population) factor. The plant part and part × population interaction was tested by the part × plant (within population) factor, and the mirid-damage and two- and three-way interactions with population and parts were tested by the residual error. Differences between populations (existing vs. transplanted mirids) and sites were somewhat confounded with differences in time of harvest and phenological growth stages of larkspur. Therefore, the difference in toxic and total alkaloids at each site was tested by paired t tests, comparing mirid-damaged and undamaged leaves and heads on the same plant.

In the 1996 study, the concentration of toxic and total alkaloids in mirid-damaged and undamaged plants was compared among locations and plant parts by GLM.

The alkaloid data from the two field studies were then combined and analyzed together in a large GLM model. Sites were tested by the plant (within site) factor. Mirid-damage and the site × damaged factors were tested by the residual error. There was a significant site × mirid-damage interaction ($P < 0.05$) for both leaves and heads. Therefore, the difference in toxic and total alkaloids in mirid-damage and undamaged parts from the same plant at each site (except Yampa) were tested by paired t tests. Yampa samples were compared by one-way ANOVA.

The variability in alkaloid concentration due to the mirid damage and the inherent variability between plants was partitioned within each population and site by a randomized block design ANOVA. The sum of squares for treatment (mirid-damaged vs. undamaged) and the sum of squares for block (individual plants) was divided by the total sum of squares to determine the proportion of variability due to the mirid and the proportion due to differences among individual plants (Coleman et al., 1987).

Nutrient components (CP and NDF) of mirid-damaged and undamaged larkspur leaves in the 1996 study were analyzed by GLM in a 2 × 3 factorial design. Mirid damage was a fixed factor and was tested by the residual error. The three sites were random factors and were tested by the damage by location interaction.

RESULTS AND DISCUSSION

Mirid-Damaged vs. Undamaged Plants in 1992, 1995, and 1996 Studies

Mirid-damaged larkspur plants in the 1992 greenhouse study were lower in the toxic alkaloid MLA than the undamaged control plants ($P = 0.07$, Table 1). The levels of MLA in the potted plants in the greenhouse are typically lower than plants in their natural populations in the field (Ralphs et al., 1997b).

Mirid-damaged leaves were lower in toxic alkaloids in the combined 1995 and 1996 data set than control leaves (5.0 vs. 6.0 mg/g, $P < 0.001$, Table 1). Total alkaloids in mirid-damaged leaves were also lower in the 1996 study (12.8 vs. 15.1 mg/kg, $P < 0.001$), but were similar in 1995 to control leaves (27.0 vs. 26.2 mg/kg, $P = 0.17$). There were few differences in toxic alkaloids in flowering heads in either year.

There were large differences in both toxic and total alkaloids between larkspur plant parts in 1995 ($P < 0.001$). Leaves contained 2.4 times more toxic alkaloids than flowering heads and 75% more total alkaloids. This is contrary to the seasonal trend in alkaloid levels in which the flowering heads and pods are generally higher in toxic alkaloids (Ralphs et al., 1997d) and total alkaloids (Ralphs et al., 1988) than leaves. There were no differences between leaves and flowering heads in the 1996 study.

There were differences in both toxic and total alkaloids between sites when averaged over mirid-damaged and undamaged plants in the 1995 study ($P < 0.05$). The 6-Mile site had higher toxic and total alkaloids in larkspur leaves and higher toxic alkaloids in heads than the other sites (Table 1). The 12-Mile site had higher total alkaloids in larkspur heads. This was probably due to differences in phenological development at the time of harvest. Both toxic and total alkaloid concentration in tall larkspur are highest in early growth and decline as the plants mature (Manners et al., 1993; Ralphs et al., 1997d). Plants harvested at 6-Mile were in the earliest phenological growth stage (early bud), and plants at 12-Mile were in the bud elongation stage when harvested, compared to the early and full flower stage at Ferron Reservoir and Duck Fork, respectively.

There were differences between locations in the 1996 study. Larkspur heads

TABLE 1. TOXIC AND TOTAL ALKALOID CONCENTRATION IN MIRID-DAMAGED AND UNDAMAGED PLANT PARTS IN EXISTING AND TRANSPLANTED MIRID POPULATIONS AT 4 LOCATIONS IN 1995 AND EXISTING MIRID POPULATIONS AT 3 LOCATIONS IN 1996

				Conc mg/g ± SE			
				Toxic Alkaloids		Total Alkaloids	
Part	Year	Mirid population	Site	Mirid-damaged	Undamaged	Mirid-damaged	Undamaged
Leaf	1992	Transplant	Greenhouse	0.2 ± 0.1b*	0.8 ± 0.3a		
	1995	Existing	Ferron	5.5 ± 0.6†	6.5 ± 0.7	22.7 ± 1.6	22.5 ± 1.4
			6-Mile	7.5 ± 0.8	7.1 ± 1.0	31.2 ± 2.8	29.4 ± 3.1
		Transplant	12-Mile	4.6 ± 0.7†	5.2 ± 0.7	26.5 ± 1.4	25.3 ± 0.9
			Duck Fork	5.2 ± 0.8†	6.4 ± 0.9	27.6 ± 1.9	27.7 ± 2.1
	1996	Existing	Mayfield	4.5 ± 0.6†	6.0 ± 0.8	19.5 ± 1.6	20.7 ± 1.7
			Salina	5.0 ± 0.6†	6.0 ± 0.7	11.1 ± 1.1†	14.1 ± 1.3
			Yampa	1.5 ± 0.1b	4.2 ± 0.5a	4.6 ± 0.4b	7.3 ± 0.1a
			Mean	5.0 ± 0.4b	6.0 ± 0.3a	21.4 ± 1.2a	21.8 ± 1.1a
Head	1995	Existing	Ferron	2.4 ± 0.3	2.2 ± 0.2	14.1 ± 1.4	12.0 ± 1.0
			6-Mile	3.3 ± 0.4	3.2 ± 0.5	16.7 ± 1.6	14.9 ± 1.6
		Transplant	12-Mile	2.1 ± 0.2	2.3 ± 0.2	18.4 ± 1.1	16.7 ± 0.7
			Duck Fork	2.9 ± 0.3†	2.1 ± 0.2	16.0 ± 0.8†	12.6 ± 0.7
	1996	Existing	Mayfield	4.1 ± 0.4	4.2 ± 0.5	15.3 ± 1.0†	18.4 ± 0.8
			Yampa	2.2 ± 0.3b	3.5 ± 0.3a	10.8 ± 0.3	8.1 ± 0.7
			Mean	2.9 ± 0.2a	2.9 ± 0.2a	15.6 ± 0.6a	14.7 ± 0.6a

*Means followed by different letters are significantly different ($P < 0.05$), as determined by ANOVA.
†Difference between mirid-damaged and undamaged parts of the same plant are significant ($P < 0.05$) as determined by paired t test.

TABLE 2. CRUDE PROTEIN AND NEUTRAL DETERGENT FIBER (NDF) IN MIRID-DAMAGED AND UNDAMAGED LARKSPUR LEAVES FROM 1996 STUDY

		Crude Protein (% ± SE)		NDF (% ± SE)	
Site	N	Mirid-damaged	Undamaged	Mirid-damaged	Undamaged
Mayfield	10	16.4 ± 0.7	18.9 ± 0.8	23.0 ± 0.5	19.7 ± 0.7
Salina	10	14.4 ± 0.4	16.7 ± 0.4	20.4 ± 0.4	17.1 ± 0.6
Yampa	6	15.5 ± 0.4	16.4 ± 0.2	21.0 ± 2.5	17.2 ± 1.1
Mean		15.4 ± 0.4b*	17.4 ± 0.4a	21.6 ± 0.4a	18.1 ± 0.5b

*Means followed by different letters are significantly different at $P < 0.05$.

and leaves at Yampa, Colorado were lowest in toxic and total alkaloids, and total alkaloids at Salina were intermediate (Table 2). Larkspur was in the full flower stage of development at all three locations when harvested. We have no explanation for why larkspur plants at Yampa were lower in alkaloids than larkspur plants at the other locations.

Existing vs. Newly Transplanted Mirid Populations, 1995

In most of our transplant studies, mirids survived, but did not increase in density or spread throughout the patch. We hypothesized that the mirid population may need to reach a critical threshold to suppress the chemical defenses of larkspur before the mirid population could continue to grow. However, there was no difference in toxic or total alkaloids in larkspur plants from existing mirid populations compared to those plants onto which the mirids were recently transplanted ($P > 0.30$). Neither was there a population $\times$ mirid-damage interaction ($P > 0.62$), which suggests that mirids did not suppress alkaloids to a greater degree in the existing population, compared to the newly transplanted population. Therefore, norditerpenoid alkaloids may not constitute chemical defenses against the mirids.

In the transplant populations, mirids caused substantial damage on the halves of plants on which they were placed ($P < 0.01$). On the mirid-damaged halves, 29% of the leaves were desiccated, 50% of the remaining leaf biomass was damaged, and 82% of the flowering heads aborted. In contrast, the undamaged halves had no desiccated leaves, only 12% of the leaf biomass was damaged, and 5% of the heads aborted from other natural causes.

This high level of damage inflicted by mirids on larkspur plants may affect the reproductive performance and long-term vigor of larkspur populations. The mirids caused the flowers to abort, thus preventing seed production. However, tall larkspur is not dependent upon seed production to maintain its population. It is a long-lived perennial forb, living up to 75 years (Cronin and Nielsen, 1979). However, leaf damage may reduce the vigor of larkspur plants, thus allowing other plants a competitive ecological advantage, and subsequently reduce larkspur's dominance in the plant community over time.

The alkaloid content of the heavily damaged leaves that had senesced remained quite high. These leaves were brown, withered, and desiccated. The toxic alkaloid concentration in these desiccated leaves was intermediate between the undamaged and mirid-damaged leaves (5.8, 5.6, and 4.9 mg/g, for undamaged, desiccated, and mirid-damaged leaves, respectively, $P = 0.04$). In contrast, during the natural maturing and senescing process, toxic alkaloids in larkspur leaves decline to less than 1 mg/g (Pfister, unpublished data). It appears that mirid-induced senescence stops alkaloid exportation and retains the alkaloids within the leaves.

Variation in Alkaloid Concentration

There was more variation in toxic and total alkaloid concentration between larkspur plants than between mirid-damaged and undamaged parts of the same plant. Toxic alkaloid concentration varied from a low of 0.6 to a high of 6.1 mg/g in heads and from 1.1 to 12.7 mg/g in leaves. Total alkaloid concentration varied from 6.2 to 26.3 mg/g in heads and from 3.4 to 47.6 mg/g in leaves. This represents a 7- to 10-fold difference in toxic alkaloids and 4- to 14-fold difference in total alkaloids. In partitioning the source of variability in the analysis of variance in the 1995 study, 77–98% of the variation in both toxic and total alkaloids in leaves was due to differences between plants, compared to only 0.01–7% due to treatments (mirid-damaged vs. undamaged plant parts). In the heads, 51–88% of the variation was due to differences between plants, but the variation due to treatment ranged from 3 to 35%. In the 1996 study, variability due to differences between plants ranged from 47 to 91% of total variability. Clearly, most of the variation in toxic and total alkaloids occurs between plants, rather than from any mirid-induced response within a plant. This is why we compared mirid-damaged and undamaged parts on the same plant.

It is generally accepted that plants evolved chemical defenses to protect against herbivory of insects (Tallany and Raupp, 1991) and browsing mammals (Bryant et al., 1992). However, the capacity of a plant to induce synthesis of secondary compounds is largely under genetic control (Coleman and Jones, 1991). Some plants can respond to damage and stress, while others cannot. Different genotypes may have different thresholds before inducing a response, and the response may vary in type and degree. Constitutive levels (initial levels) may vary greatly between individual plants and thus mask response to treatments.

Nutrients

Mirid-damaged larkspur leaves were lower in CP and higher in NDF than undamaged plants ($P < 0.01$, Table 2). This supports the theory that mirids are extracting cell solubles from larkspur leaves, leaving a higher proportion of fiber from the cell walls. Pfister et al. (1988b) reported that tall larkspur was a nutritious forage. Crude protein in its leaves declined from a high of 27% in early growth, to 9% at the end of the season. NDF remained between 10 and 15% throughout the season. The decline in nutrients and increase in fiber may contribute to the reduced palatability of mirid-damaged larkspur to cattle. In a previous feeding trial, cattle preferred undamaged larkspur compared to mirid-damaged larkspur (Ralphs et al., 1997a). In that study, mirids were not present on larkspur plants fed to cattle (they were shaken off when the plants were harvested), and recent rains had washed all their fecal material off the leaves.

Therefore, taste, physical texture, and nutrient quality were the only factors influencing palatability. In natural larkspur populations infested with mirids, the physical presence of mirids and the accumulated fecal material on the leaves probably would further reduce larkspur's acceptability to cattle.

CONCLUSIONS

It is readily apparent that damage from larkspur mirids did not induce norditerpenoid synthesis. Rather, there was a slight decline in toxic alkaloid concentration in larkspur leaves. Ralphs et al. (1997b,c) reported that the relative concentration of both toxic and total alkaloids increased in larkspur plants stressed by shade, photosynthesis inhibition, and herbicide treatments. They speculated that nonstructural carbohydrates and other soluble compounds declined during these stresses, thus increasing the relative concentration of a constant amount of alkaloids. The response of toxic alkaloids to feeding stress from mirids in this study was opposite. The relative concentration of toxic alkaloids declined in mirid-damaged leaves, along with a decline in N and an increase in fiber. Perhaps the mirids extracted toxic alkaloids from larkspur, thus accounting for the slight decline in concentration. Further research is being conducted to determine if the toxic norditerpenoid alkaloids are sequestered by the mirids and excreted in their fecal material. It appears that damage from larkspur mirids does not induce norditerpenoid alkaloid synthesis as a chemical defense in tall larkspur.

Even though mirids reduced toxic alkaloids in larkspur leaves, it may not have been sufficient to reduce the threat of poisoning cattle. Toxic alkaloid levels above 3 mg/g pose a threat to grazing cattle (Ralphs et al., 1997d). Only the mirids at Yampa, Colorado, reduced toxic alkaloids below the toxic threshold. It is unlikely that damage from the larkspur mirid will universally reduce larkspur toxicity below the toxic threshold. In fact, if damaged leaves senesce and retain the alkaloids, rather than translocating them out during the natural senescing process, toxicity of larkspur may be arrested at a high level. The benefit of the larkspur mirid as a biological tool rests solely in its ability to damage larkspur and render it unpalatable to cattle.

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EVALUATION OF SWAB AND RELATED TESTS AS A BIOASSAY FOR ASSESSING RESPONSES BY SQUAMATE REPTILES TO CHEMICAL STIMULI

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Abstract—The ability of squamates to detect chemical cues from adaptively important sources including prey, predators, and conspecifics has been tested frequently by presenting stimuli on cotton-tipped swabs or ceramic tiles. In many such studies the primary response variable is tongue-flicking, which is widely interpreted to indicate sampling for vomerolfaction. I review the basic experimental method and consider limitations regarding its application and interpretation and ways to overcome them. Effects of experimenter proximity and the assumed invisibility of chemical stimuli are considered, as are use of cologne as a pungency control, senses used in making chemical discriminations, and interpretation of results when there are no significant response differences among stimulus classes. Although the assumption that tongue-flicking reveals vomerolfactory sampling and the necessity of an intact vomeronasal system for normal responses to pheromones have been demonstrated where tested, very few species have been examined. In some squamates for which these assumptions have not been examined experimentally, especially eublepharid geckos, attacks on swabs bearing prey chemicals and performance of antipredatory displays in response to predator chemicals occur with no prior tongue-flicking. Not only are assays based on tongue-flicking useless in such cases, but the discriminations are likely based on olfaction. Issues specific to the study of responses to prey chemicals, predator chemicals, and pheromones are discussed. For many purposes, swab tests provide rapid, conclusive assays of ability to respond differentially to biologically relevant stimuli. However, other methods may be superior for studying some responses, and swab tests are not always applicable.

Key Words—Tongue-flicking, behavior, chemical senses, Squamata.

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INTRODUCTION

Tongue-flick rates in response to cotton swabs bearing chemical stimuli have been used for 60 years to assess responses by squamate reptiles to a variety of biologically important stimuli (Wilde, 1938; Burghardt, 1967). Burghardt (1980) argued that the method is an important source of comparative data on chemosensory responses based on an easily observed and recorded behavior correlated with the function of a specific sense, vomerolfaction. It and related methods using measures of tongue-flick rates have been widely used in comparative studies of response to prey chemicals and pheromones, notably in the laboratories of Alberts, Arnold, Burghardt, Chiszar, Cooper, Duvall, and Halpern (cited below). Use of tongue-flick rates as a response measure avoids the impracticality and ethical concerns of invasive surgical intervention to test chemosensory roles (Burghardt, 1980) while providing more quantitative information than can be obtained from such methods.

Although swab tests have been used to study responses to chemical cues arising from prey and predators, as well as pheromones, relatively little attention has been paid to possible interpretive difficulties. Here, I first describe tongue-flicking, its function, and its association with vomerolfaction, and then I describe the swab method and its rationale, discuss some difficulties with its implementation and interpretation, and consider its utility.

TONGUE-FLICKING: FORM AND SENSORY ASSOCIATION

Lingual protrusion has several forms in squamate reptiles, including spectacle wiping, defensive displays, labial licking, and tongue-flicking (Simon, 1983; Cooper, 1994a; DePerno and Cooper, 1996). In typical tongue-flicking the tongue is projected outward and down from the mouth and is returned by sweeping upward and back into the mouth (Gove, 1979). Molecules on substrates contacted by the ventral foretongue adhere to its surface, which in snakes has a type of cell whose surface is a microscopically faceted pore system (Morgans and Heidt, 1978; Mao et al., 1991) that might enhance adherence. Adherent molecules are carried into the mouth as the tongue returns to its resting position. Radioactively labeled chemicals on substrates contacted by the tongue during tongue-flicking have subsequently been detected on the sensory epithelium of the vomeronasal organ (Graves and Halpern, 1989). The tine tips of the anteriorly split squamate tongue do not carry chemicals into the vomeronasal ducts, but the chemicals are transferred to projections on the ventral floor of the mouth and appear from there to gain entry to the ducts located above (Gillingham and Clark, 1981; Young, 1993). Tongue-flicking thus serves to gather chemical samples for delivery to the mouth and indirectly via the vomeronasal ducts to

the vomeronasal organs. Taste buds are present in the oral mucosa and on the tongues of many squamates (Schwenk, 1985), raising the possibility that gustation is important in chemical discriminations mediated by tongue-flicking (but see below).

Some other forms of lingual protrusion are readily distinguishable from tongue-flicking and obviously have different functions (Gove, 1979). In spectacle wiping, prominent in gekkonid and xantusiid lizards (Simon, 1983; Greer, 1985), the tongue is protruded posterolaterally and swept over the spectacle of the eye. This behavior appears to be a form of grooming. In defensive contexts the tongue of some snakes is protruded and waved slowly (Gove and Burghardt, 1983) or held nearly rigid; such species often have tongue coloration that is bright or contrasts with the body color (Mertens, 1955; Pitman, 1974; Gove, 1979). Labial licking has two forms that in practice are distinct, but might be part of a continuum (Cooper et al., 1996a; DePerno and Cooper, 1996). After swallowing, many squamates wipe their labial scales with their tongues. A substantial portion of the tongue is protruded sufficiently to wipe the labials along one side. Like spectacle wiping, this behavior appears to represent grooming. Another form of labial licking occurs in which the squamate extends only the tip of its tongue, bringing it into contact with the anterior labials. This form of labial licking appears in response to prey chemicals presented on swabs and may have a chemosensory sampling function, such as resampling chemicals either on the tongue or labials (Cooper et al., 1996a; DePerno and Cooper, 1996). The latter form of labial licking is often performed rapidly and repeatedly, in contrast to labial licking after swallowing. At a finer level of analysis, features of tongue-flicks differ among contexts. For example, in juvenile *Thamnophis sirtalis* the tongue was extended further while exploring an empty box than while investigating a food item and still further in the presence of a conspecific; tongue-flicks were fastest during investigation of food (Gove and Burghardt, 1983).

In swab tests, only typical tongue-flicking and the limited-extension, anteriorly directed labial licking are likely to occur. Some species perform primarily tongue-flicks (e.g., *Thamnophis sirtalis*), whereas others, such as *Eublepharis macularius* and *Eumeces laticeps* perform both types frequently (Cooper, unpublished observations). In most of my studies, I have counted both of these behaviors as tongue-flicks because the type of labial licking in question appears to be an abbreviated form of tongue-flicking. From an anthropomorphic perspective, both of these types of lingual protrusion appear to be investigatory.

Because tongue-flicking serves to gather chemical samples for delivery to the vomeronasal organ, tongue-flicking may indicate involvement of vomerolfaction. However, neither exclusive function nor participation of vomerolfaction can be inferred safely without critical experimentation involving selective blocking or elimination of nasal chemical senses (Halpern, 1992). In the few squamate

species that have been studied, elimination of vomerolfaction blocks social and foraging behaviors associated with elevated tongue-flicking rates, whereas elimination of olfaction permits them to continue (reviewed by Halpern, 1992). Because lingually mediated prey chemical discrimination is abolished in an iguanid lizard (Cooper and Alberts, 1991) and a scincid lizard (Graves and Halpern, 1990) by blocking the vomeronasal ducts and in garter snakes by severing the vomeronasal nerves (Halpern and Frumin, 1979), the discrimination requires vomerolfaction. Because the responses are abolished in the absence of functional vomerolfaction, neither olfaction nor lingual gustation are sufficient to support the behavioral discriminations.

Tongue-flicking appears to be an important indicator of vomerolfactory investigation, but it is not necessary to assume that tongue-flicking is evidence of vomerolfactory activity for tongue-flick rate to be a useful assay in experimental studies of discriminatory capabilities or prey chemical preferences. Even if multiple senses are used for a given discrimination, tongue-flicking by itself may be adequate to detect the discrimination. It is conceivable that some species make olfactory discriminations that are revealed by tongue-flicking for vomerolfactory investigation of stimuli initially detected by olfaction, even if vomerolfaction provides no further information. However, elimination of other behavioral responses such as predatory attack when vomerolfaction is blocked indicates that vomerolfaction is needed to make the discriminations. In other cases tongue-flicking may be absent when discriminations are made or may play a secondary role not permitting detection of the discrimination. Thus, although differential tongue-flick rates indicate discrimination regardless of the senses used, their absence does not necessarily indicate a lack of discriminatory ability.

BASIC EXPERIMENTAL APPROACH

In laboratory studies in which chemical discriminations are assessed by tongue-flicking directed to swabs, an animal usually responds in a given trial to a single swab bearing stimuli belonging to one of several categories of experimental and control stimuli (e.g., Burghardt, 1967; Cooper and Burghardt, 1990), but simultaneous presentations have also been employed in nonswab tests (e.g., Reformato et al., 1983). The method summarized here is discussed in more detail by Burghardt (1970a) and Cooper and Burghardt (1990) and is outlined in Figure 1. To start a trial, a swab is moved to a position just anterior to the animal's snout and held there for a fixed interval, during which the animal may respond. Experimental designs are typically either independent groups designs, in which an individual responds to only one stimulus type, or repeated measures (= randomized blocks) in which each individual responds to all stimuli in either a randomized or counterbalanced sequence. The same stimuli also may be tested

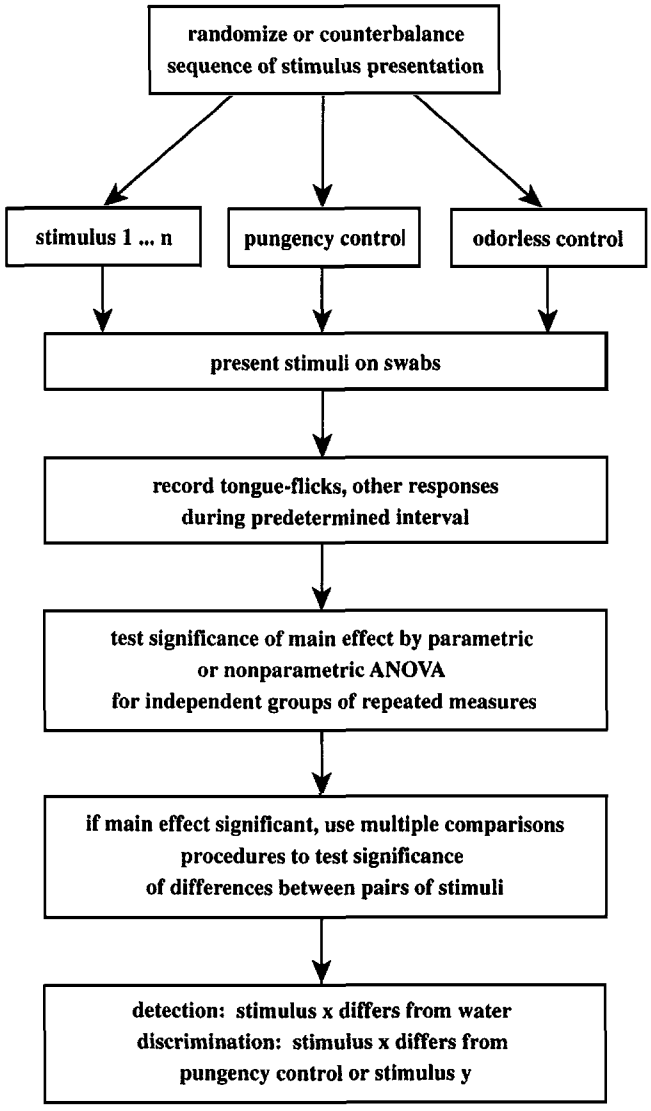

FIG. 1. Experimental paradigm of swab tests as often used in studies of lingually mediated prey chemical discrimination.

more than once per individual. In the most basic application, significance of differences among stimulus sources in numbers of tongue-flicks is tested over a preset response interval.

If the assumptions of parametric tests are satisfied, analysis of variance is used if three or more stimulus types are tested and the *t* test is used if there are only two (Zar, 1984). If assumptions of parametric tests are violated or the investigator prefers the ease of calculation and lack of restrictive assumptions, nonparametric analysis of variance, Wilcoxon matched-pairs signed-ranks tests, or Mann-Whitney U tests can be used (Zar, 1984). When responses to three or more stimulus sources are tested, the nonparametric analysis may be conducted by Kruskal-Wallis one-way analysis of variance for experiments with independent groups designs or Friedman two-way analysis of variance for experiments with randomized blocks designs. For either type of nonparametric analysis of variance, multiple comparisons procedures can be used to test for significance of differences among pairs of means if the main stimulus effect is significant (Zar, 1984).

If tongue-flick rates differ significantly among conditions or treatments, it is concluded that differences were detected among stimuli. Depending on the overall pattern of significance among treatments, it may be concluded that the animals not only detected some stimuli, but discriminated among them. For example, in studies of prey chemical discrimination, I have typically used three stimuli: prey chemicals, an odorless control (deionized or distilled water), and a pungency control (cologne), the latter to account for responses to an odorous, but irrelevant stimulus. If there is a significant difference between responsiveness to the odorless control and another stimulus, it may be concluded that the other stimulus was detected. If the tongue-flicking rate differs significantly between the pungency control and prey chemicals, it may be concluded that the animals discriminated between the cologne and the prey chemicals. This conclusion rests on the assumption that the squamates detected the cologne. In other studies, stimuli from multiple potential food sources act as pungency controls (e.g., Burghardt, 1969, 1970b; Cooper and Alberts, 1990; Cooper et al., 1990).

METHODOLOGICAL AND INTERPRETIVE ISSUES

Experimenter Presence and Proximity

Because swabs are typically hand-held, the experimenter's hand is very close to the lizard. Because human presence may elicit escape behavior or defensive immobility, I have typically held lizards in captivity for a month or longer to allow acclimation to laboratory conditions and the proximity of human beings, including hands, before conducting swab tests. Lizards and snakes in a

wide variety of taxa respond well in swab tests of prey chemical discrimination (reviewed by Cooper, 1994b). The method has also worked well for tests of responses to pheromones in representatives of Scincidae (e.g., Cooper and Vitt, 1984a, b), Gerrhosauridae (Cooper and Trauth, 1992), and Helodermatidae (Cooper, unpublished data), and for tests of responses to predator chemical cues in a scincid lizard (Cooper, 1990a).

However, some species tongue-flick little or not at all during swab tests (Cooper, 1989a, 1994a). Even in species that tongue-flick readily in such tests, some individuals are completely unresponsive. For species in which many individuals do tongue-flick, I have eliminated data for individuals that did not tongue-flick because they had no opportunity to discriminate based on chemicals sampled by tongue-flicking. Use of ingestively naive neonatal animals to help eliminate variation due to experience has been extensive in studies of squamate chemosensory behavior (e.g., Arnold, 1978, 1981; Burghardt, 1967, 1969, 1970b). Prescreening animals for responsiveness in the experimental context is another useful technique (e.g., Reformato et al., 1983). For species and individuals that do not tongue-flick, it is unclear whether lack of responsiveness indicates an inability to discriminate among stimuli, a consistent absence of chemosensory sampling by tongue-flicking by the individual or species, or a defensive response. In some species, such as crevice-dwellers, the experimental context of an open cage may be inappropriate for study of food-related behaviors. Fleeing is obviously defensive, but many squamates remain immobile, perhaps either to maintain crypsis or to avoid triggering attack by the potential predator after moving.

Several alternative procedures may be tried for species that do not tongue-flick reliably in swab experiments in which the experimenter is visible and close to the animals (Table 1). One modification that worked in a study of pheromonal communication in *Eumeces inexpectatus* (Cooper and Vitt, 1986a) was to increase the distance between lizard and experimenter by tying the applicator stick bearing the swab onto a thin metal rod and holding the rod at arm's length during stimulus presentations. Tongue-flicking may also be observed from a distance through binoculars (A. Lewis, personal communication).

Perhaps a better approach is to minimize or eliminate visual detection of the experimenter. Burghardt (1975) advocated reduction of disturbance by having the experimenter sit in the shade above a cage lit from below on the opposite side. Visual detection of the experimenter could be eliminated readily by using a small port in the side of the cage allowing entry only of the swab and its applicator stick. One side or the top of the cage could consist of one-way glass. Modification of the swab method to permit access through ports has not been tried, but seems promising for collection of comparative data. Alberts (1989) used blindfolding to study responses by an iguanid lizard to pheromonal stimuli

TABLE 1. DIFFICULTIES POSSIBLY ARISING IN STUDIES OF TONGUE-FLICKING TO STIMULI PRESENTED ON COTTON-TIPPED APPLICATORS: CAUSES AND SUGGESTED SOLUTIONS

Problem	Cause	Solution
No tongue-flicking	Experimenter proximity, antipredatory immobility	Acclimate to laboratory and experimenter presence; lengthen applicator; hide experimenter by lighting, introduce swab through port; blindfold animals; present stimuli on tiles, view from blind or videotape remotely
	Context inappropriate	Change context
No tongue-flicking by a few individuals	Experimenter proximity, defensiveness, other	Discard data for those individuals
Response to visual cues	Stimuli visible in near UV	Screen for fluorescence, blindfold if necessary
Suppression of tongue-flicks or avoidance of cologne	Noxious if undiluted	Dilute cologne, use other pungency control (potential foods)

presented on tiles. Blindfolding seems to have great promise for use with the swab method due to elimination of visual disturbance by the experimenter and of visual cues from the stimuli that might complicate investigations of chemosensory ability.

In studies of prey chemical discrimination and pheromonal communication, I have sometimes eliminated visual cues by presenting the stimuli on ceramic tiles placed on cage floors and have observed tongue-flicking and other responses from a blind (Cooper et al., 1996b; unpublished data). The tile method is somewhat more time-consuming, in part because the tiles lack visual cues that might elicit chemosensory investigation, even when trials are conducted at sites other than the home cage to stimulate chemosensory explorations. Furthermore, tongue-flick rates to tiles may not be directly comparable to those in the swab method. However, it works in some cases in which the simple swab method fails. As a last resort, squamates may be grasped and swabs bearing various stimuli inserted into their mouths, tongue-flicks being recorded after they are released (Cooper, 1992a). Halpern and colleagues (Reformato et al., 1983; Kirschenbaum et al., 1986) have long used a very similar technique in which two chemical stimuli are presented simultaneously on Coplin jar covers on a tray that can be placed in the animal's home cage. A related technique used in Halpern's laboratory is presentation of chemical stimuli on plastic worm bits

glued to plastic dishes that can be placed in the snakes' home cages (Wang et al., 1993).

In a recent study of prey chemical discrimination by a lizard (*Xenosaurus platyceps*) (Cooper et al., 1998) that rarely emerges fully from crevices in rocks, adults did not tongue-flick swabs at all when tested in bare cages. When tested in crevices, they exhibited lingually mediated prey chemical discrimination. It is likely that antipredatory immobility inhibited tongue-flicking outside the crevices, but it is also possible that the context was inappropriate for chemosensory investigation. Testing should be conducted in settings where feeding or other behaviors being investigated are known to occur. In almost all of the studies in Burghardt's and my laboratories, tests have been conducted in the home cages.

Whenever squamates fail to respond to food stimuli by tongue-flicking or fail to exhibit prey chemical discrimination, it should be established that they will eat in the experimental situation at the time of or immediately following testing. Halpern and Frumin (1979) used this technique after testing responses of garter snakes to prey chemicals on swabs to ascertain whether they would attack and ingest earthworm bits.

Invisibility of Chemical Stimuli

The stimuli must be visually indistinguishable to the animals being tested if the effects of chemical stimuli are to be experimentally isolated. In almost all studies conducted to date, it has been assumed that swabs that appear identical to people also appear identical to squamates. However, at least some squamates can see ultraviolet light invisible to the human eye (Alberts, 1989; Fleishman et al., 1993). Thus, it is possible that some responses that have been interpreted as chemosensory are actually visually mediated. This is especially likely to be the case for pheromonal deposits that are conspicuous in ultraviolet wavelengths (Alberts, 1989).

Screening of stimuli for fluorescence in the near ultraviolet might be a useful procedure for identifying stimulus differences that might be visually detectable to squamates. However, so little is known about ultraviolet visual capacities and their taxonomic variation in squamates that it is not clear whether or when such screening is needed for all sorts of stimuli. At present, ultraviolet vision is known to aid detection of pheromone deposits on substrates in only one iguanian lizard species (Alberts, 1989), but this phenomenon is likely to be much more widespread, as hinted by the visual attraction of a phrynosomatid species to pheromone deposits (Duvall et al., 1987). When the stimuli are expected to differ at the near ultraviolet wavelengths visible to lizards, swab tests could be adapted simply by blindfolding or otherwise blocking vision, as Alberts (1989) did when presenting stimuli on small ceramic tiles held in front of lizards.

Pungency Control

To ascertain that squamates respond differentially to detected chemical stimuli, cologne is often used in studies of responses to prey chemicals (numerous papers cited in Cooper, 1994c, 1995a) and pheromones (Cooper and Vitt, 1986b). Dial and Schwenk (1996) suggested that cologne is an inappropriate pungency control for studies of response to prey because it may be an irritant as well as an odorant. They further noted that in their study of responses to predator chemicals by *Coleonyx brevis*, labial licking occurred only in response to cologne and implied that this labial licking might represent cleaning after exposure to an unpleasant stimulus.

It seems reasonable to assume that squamates detect cologne (presumably by olfaction) even if tongue-flick rates in response to cologne do not differ significantly from those in response to the odorless control. In my first pilot work, I tried undiluted cologne, but found it to be noxious to lizards as well as to me. *Coleonyx variegatus* and some other lizards blink and withdraw from swabs bearing undiluted cologne, suggesting that it does irritate eyes and perhaps the nasal and oral mucosas. In published studies I have diluted the cologne sufficiently that it had a mild odor to me and did not cause my nostrils to itch or eyes to burn when held close to them. This procedure, although informal, has been effective. It could readily be improved by using a standard dilution. Observation of lizards reveals no indication that sufficiently diluted cologne is noxious to them, in contrast to withdrawal and wiping of the labials on the substrate sometimes elicited by undiluted cologne.

In my studies of responses to prey chemicals by the eublepharid geckos *Coleonyx variegatus* (Cooper, unpublished data) and *Eublepharis macularius* (Cooper, 1995b), both labial licking and tongue-flicking (pooled in the published paper) occurred frequently in response to cologne as well as cricket stimuli. With the diluted cologne used, there was no indication of facial wiping with the tongue or wiping the labials that might indicate response to a noxious stimulus. Reasons for response differences in closely related taxa in studies in my laboratory and Dial's laboratory are uncertain. One possibility is that the colognes used, Paco Rabanne (Dial and Schwenk, 1996) vs. Mennen Skin Bracer or English Leather have very different effects on the lizards. The contrasting lack of tongue-flicking by *C. variegatus* in a study by Dial et al. (1989) and frequent tongue-flicking in my unpublished study in response to cricket chemicals is unexplained. The most likely explanation is that volatiles in aqueous solutions of ground crickets (Dial et al., 1989) elicited attack, whereas the stimuli from intact crickets in my study elicited investigative tongue-flicking prior to attack.

In a series of swab studies of prey chemical discrimination, I found no difference in numbers of tongue-flicks elicited by cologne and water in a chamaeleonid (Cooper, 1989a), a crotaphytid (Cooper et al., 1996c), an iguanid

(Cooper and Alberts, 1990, 1991), a phrynosomatid (Cooper, 1989a), a polychrotid (Cooper, 1989a), a tropidurid (Cooper and DePerno, 1993), a scincid (Cooper and Vitt, 1989), a cordylid (Cooper and Van Wyk, 1994), a gerrhosaurid (Cooper, 1992b), lacertids and teiids (Cooper, 1990b, 1991a), an anguid (Cooper, 1990c), a helodermatid (Cooper, 1989b), a varanid (Cooper, 1989b), and a pythonid (Cooper, 1991b). Ten of these species exhibited elevated responses to prey chemicals. Diluted cologne, although detectable, did not elicit pronounced investigation by tongue-flicking (or labial-licking) in most taxa.

Adequately diluted cologne appears to be a suitable control for response to an odorous substance in most squamates. It is clearly detectable by olfaction, but the absence of elevated tongue-flicking provides no evidence that it may be detected by vomerolfaction. Because the reptilian vomeronasal system responds to volatile as well as nonvolatile substances (Shoji and Kurihara, 1991), volatile molecules and any larger molecules in the cologne might be detected by vomerolfaction when sampled lingually, but do not elicit elevated tongue-flicking.

Although cologne has proven useful, I agree with Dial and Schwenk (1996) that some other types of pungency controls are better. A stimulus that is known to be both nonnoxious and detected by the vomeronasal system would provide the best pungency control. Foods eaten and known to elicit chemosensory sampling by tongue-flicking in related species, but not eaten by the species being tested, seem a strong possibility. Unfortunately, many lizards are food generalists that consume a wide variety of arthropods and other invertebrates. For such species, in which potential animal foods not eaten tend to be chemically repellent, plant surface chemicals might prove useful.

For taxa that have somewhat specialized diets, variation in response strength among actual foods and potential prey species that are not usually eaten, but are not noxious to other consumers, can provide valuable pungency controls. For example, the colubrid snake *Masticophis flagellum*, a specialist predator of snakes and lizards, tongue-flicked at higher rates in response to chemical stimuli from several species of lizards and snakes than to similarly prepared stimuli from potential prey species belonging to several other taxa (Cooper et al., 1990). Perhaps the best pungency controls are foods that are acceptable, but less preferred than other foods being tested as sources of chemical stimuli. These controls have been widely used in studies of garter snakes, natricine colubrids of the genus *Thamnophis* (Burghardt, 1969, 1970b; Arnold, 1981), and provide the most convincing evidence that squamates make fine discriminations among possible chemical food stimuli.

For studies of responses to pheromones and predator chemicals, natural pungency controls are preferable to cologne or other artificial stimuli. Differential responses to pheromones from conspecifics and members of closely related species and to chemical stimuli from related predatory and nonpredatory species

eliminate the need for pungency controls when it can be assumed that the stimuli are all readily detectable.

Senses Used

Despite the close relationship between tongue-flicking and vomerolfaction and the common use of tongue-flicking to assess chemosensory responses to various stimuli, olfaction may be important to squamates both for activation of vomerolfactory sampling by tongue-flicking and for making important chemosensory discriminations independent of vomerolfaction. Because the olfactory system responds solely to highly volatile substances of low molecular weight and low information content, whereas the vomeronasal system responds to nonvolatile substances of high molecular weight and high information content (Halpern, 1983; Shoji and Kurihara, 1991), it has been hypothesized that detection of volatiles by olfaction activates tongue-flicking to collect samples to be analyzed in detail by vomerolfaction (Cowles and Phelan, 1958). This hypothesis has received some experimental support (e.g., Duvall, 1981).

The squamate species in which effects of selective blocking of olfaction and vomerolfaction have been studied have less highly developed olfactory systems than some other squamates. It is possible, even likely, that olfaction is more important in making socially and trophically important chemical discriminations in taxa having stronger olfactory senses. The most highly developed olfactory systems, as indicated by the abundance of chemoreceptor cells in the olfactory epithelium, occur in Gekkonidae, Eublepharidae, Xantusiidae, and Amphisbaenia (Gabe and Saint Girons, 1976; Cooper, 1996a). Olfaction appears to be relatively important, but somewhat less well-developed in Lacertidae, Teiidae, Gerrhosauridae, and Scincidae, and weaker in the remaining families (Gabe and Saint Girons, 1976; Cooper, 1996a).

Schwenk (1993) adduced several lines of evidence to suggest the importance of olfaction in geckos. Behavioral evidence indicates that eublepharid geckos can respond appropriately to biologically significant odors of predators (Dial et al., 1989) and prey (Dial et al., 1989; Brillet, 1990) without tongue-flicking. In my own studies of prey chemical discrimination, I have observed *Eublepharis macularius* and *Coleonyx variegatus* (Cooper, unpublished observations) to bite cotton swabs bearing prey chemicals without first tongue-flicking or contacting the swabs. Such bites are less frequently directed to swabs bearing an odorous control substance (cologne) and more rarely to swabs bearing only water. I have made similar (unpublished) observations at high frequency for the gekkonid *Phelsuma madagascariensis* and the xantusiid *Xantusia vigilis* and at lower frequencies in members of several other lizard taxa.

Many gekkonoid lizards perform rapid buccal expansion and contraction visible in the gular region. Dial and Schwenk (1996) have presented evidence

suggesting that such buccal pulsing is a mechanism of olfactory sampling correlated with olfactory discrimination of chemical predator stimuli without tongue-flicking or vomerolfaction in the eublepharid gecko *Coleonyx brevis*. Thus, in some geckos and perhaps certain other squamates, olfaction alone may be sufficient to make adaptively significant discriminations or behavioral decisions. Because *X. vigilis* and *P. madagascariensis* bite swabs bearing cologne as well as those bearing prey chemicals without first tongue-flicking (Cooper, unpublished observations), attack may be triggered by olfactory cues having some general properties often associated with prey rather than by properties specific to a particular prey type.

For species that make behavioral decisions regarding performance of antipredatory displays or attack on potential prey based on olfactory discriminations, tongue-flicking may or may not be adequate to assess chemosensory discrimination. It may be adequate if vomerolfaction is also used to assess the chemicals, requiring sampling by tongue-flicking. However, if the decision is based primarily on olfaction, few or no tongue-flicks may occur.

Tongue-flicking cannot be counted on to detect chemical discriminations based on olfaction, but a wide range of chemical discriminations are accompanied by tongue-flicking in a wide range of squamate taxa. Whether or not tongue-flicking is essential, discriminations are often apparent from differential tongue-flicking rates (citations above, reviewed in Cooper, 1994b). Whatever senses are involved, tongue-flicking is a good indicator of chemosensory investigation and discrimination among stimuli. Whether the swab method is adequate to detect discriminations depends on the biology of the particular species and the particular discrimination being studied. The presence of visual cues such as size and movement, which are known to affect predatory behavior by squamates (Burghardt, 1973; Cooper, 1981; Nicoletto, 1985), might affect investigation of chemical cues.

Absence of Significant Differences

In a typical study using swab tests, the interpretation of significant differences among stimulus classes is straightforward, but absence of significant differences is more difficult to interpret (Table 2). Lack of significant differences might indicate that the animals detect no differences among stimuli. Persistent tongue-flicking often appears to be an attempt to localize the source of chemical stimuli in the absence of visual cues. However, if no significant differences among stimuli are detected, one cannot be certain that tongue-flick rate is correlated with either detection or recognition of chemical stimuli.

Some species might detect differences, but not respond by differential tongue-flicking if there is no compelling reason for prolonged chemosensory investigation. For example, the absence of sex discrimination revealed by tongue-

TABLE 2. REASONS FOR LACK OF EXPERIMENTAL DETECTION OF DISCRIMINATION INDICATED BY DIFFERENTIAL TONGUE-FLICK RATES

Actual condition	Cause	Statistical/experimental issues
No discrimination occurs	Not capable of discrimination	Power analysis can show that discrimination would have been detected given adequate sample size
	Capable, but visual or other cues needed to elicit tongue-flicking are missing	Power analysis would be misleading, researcher must provide the needed cues
Discrimination occurs	Tongue-flicks to discriminated stimuli do not occur at differential rates	Discrimination not detectable by tongue-flick rate
	It occurs rapidly, and continued tongue-flicking is disadvantageous due to foraging or antipredatory behavior	Discrimination not detectable by tongue-flick rate
	Sample size is too small to detect it	Increase number for adequate statistical power

flicking in female *Eumeces laticeps* (Cooper and Vitt, 1984b) and *Eublepharis macularius* (Cooper and Steele, 1997) may reflect the lack of a need to locate mates by scent-trailing (Cooper and Vitt, 1986c) rather than a true lack of ability to discriminate.

In some circumstances there might be direct selection against continued tongue-flicking after a rapid discrimination. This could occur in studies of prey chemical discrimination by species that rely on immobility to maintain crypsis, including many ambush foragers (Vitt and Congdon, 1978; Vitt and Price, 1982). Even if ambush foragers can recognize prey chemicals by one or a few tongue-flicks, their hunting response might be adoption of an immobile ambush posture in which further tongue-flicking is suppressed due to detectability by prey and predators (Cooper, 1995a).

Other factors that might prevent detection of discriminations in tongue-flicking studies are defensiveness, as discussed above, and absence of appropriate visual stimuli that might be needed to induce chemosensory sampling by tongue-flicking or to maintain tongue-flicking after the initial sampling. Although the latter is not known to occur, an analogous effect may occur when swabs smaller than acceptable prey size are used as chemical stimulus sources. When

I presented mouse chemicals to *Python regius* on cotton-tipped applicators in pilot tests, none bit the swabs. Some individuals did bite when the same stimuli were presented on swabs the size of mice (Cooper, 1991b).

Given these limitations, it is not always possible to tell whether absence of significant differences indicate inability to discriminate among stimuli or merely a lack of visible manifestation by tongue-flicking. Nevertheless, it is possible to use the concept of statistical power (Winer, 1962; Sokal and Rohlf, 1995) to assess whether the absence of discrimination is a reliable phenomenon. Based on results of previous work with the same experimental design, one can estimate the likelihood that true differences in tongue-flick rates among stimuli would be statistically significant. When sample size is adequate to detect the difference in more than 95% of replications, it may be concluded that a lack of significant difference indicates a true absence of difference in tongue-flick rates among stimuli. Such analysis does not address the issue of ability to discriminate among stimuli, but permits inferences regarding relative tongue-flick rates elicited by the stimuli.

APPLICATION TO PREY, PREDATOR, AND PHEROMONAL STIMULI

Tongue-flicking increases in situations requiring acquisition of chemically coded information, such as encounters with unfamiliar conspecifics (Cooper, 1996b) and novel situations (Defazio et al., 1977). It is this role of tongue-flicking in chemosensory investigation that makes tongue-flicking a useful assay for many fundamental aspects of squamate behavior. Relationships between stimulus strength and tongue-flick rate, as well as effects of motivational factors such as hunger and reproductive condition may be studied directly by the swab method. Such studies can demonstrate correlations between stimuli and tongue-flick rates and strengthen inferences that tongue-flicking is used for chemosensory investigation of particular classes of stimuli. Because tongue-flicking behavior may be modified by food chemicals, aversive substances, and association with aposematic visual stimuli, it can also be used to study effects of experience on chemosensory investigation and preference (Fuchs and Burghardt, 1971; Burghardt et al., 1973; Arnold, 1978; Burghardt, 1992; Terrick et al., 1995).

Tongue-flick rates measured by the swab or other methods are often used in studies of prey chemical discrimination, detection of predator chemicals, and response to pheromones. Significant differences in tongue-flick rates permit inferences about the adaptive significance of some discriminations, but may be less reliable in other cases. Investigators have relied on multiple responses to demonstrate discriminations when tongue-flicking alone is unconvincing and when other responses to chemical stimuli are more convincing and/or interfere

with further tongue-flicking. For example, antipredatory displays and open-mouthed predatory attack on the stimulus source are more cogent evidence than investigation by tongue-flicking, and both may reduce tongue-flicking. In studies of prey chemical discrimination and pheromonal communication, data on tongue-flicks and attacks on swabs or performance of behavioral displays after tongue-flicking tiles have been used to form composite variables indicating response strength. These and other issues specific to studies of discriminations involving prey chemical stimuli, predator chemical stimuli, and pheromones are discussed next.

Prey

Many squamate species tongue-flick or bite swabs bearing prey chemicals more frequently than swabs bearing control substances, demonstrating a differential response to food chemicals and supporting the inference of chemosensory investigation of a potential food source. The predatory attacks on swabs bearing prey chemical stimuli provide strong evidence that the chemical cues are recognized as food stimuli. Data may be analyzed in several ways, most of which include tongue-flick rates (Table 3). However, biting interferes with tongue-flicking during the actual attack and handling of the swab and perhaps afterwards. Therefore, investigators have typically terminated trials as soon as biting occurs (Burghardt, 1967; Cooper and Burghardt, 1990). Especially if the bite has a very short latency, the number of tongue-flicks may be as low as one. This is clearly a poor representation of response strength to the food stimuli.

The most frequently used method of analyzing such data is to calculate a composite variable, the tongue-flick attack score (TFAS), which weights biting more heavily than tongue-flicking and gives additional weight to bites occurring at short latency. Although the amalgamated tongue-flicks and bites at first thought seem very much like apples and oranges, both the behaviors and the short latency are considered to represent response strength to the feeding stimulus. Both in practice and for theoretical reasons, Cooper and Burghardt (1990) concluded that TFAS and its modifications give the best indication of response strength among numerous variables studied.

A major reason for using TFAS is that biting by a few individuals may obscure differences in tongue-flick rates, but not be frequent enough for detection of differences among stimuli in bite frequency or latency. This can be a serious problem because available sample size and percentage of individuals biting are often small. Cooper and Burghardt (1990) recommended use of TFAS in conjunction with separate data on tongue-flick rate, number of individuals attacking swabs, and latency to attack. If desired and where feasible, sample size may be increased to detect significant differences in frequency of biting and tongue-flick rates.

TABLE 3. APPLICABILITY OF SWAB AND RELATED METHODS TO DETECT SQUAMATE CHEMICAL DISCRIMINATIONS OF PREY, PREDATORS, AND PHEROMONES

Stimuli	Advantages and disadvantages	Variables	Possible limitations (solutions)
Prey	Quick, standardized, works well for many taxa	Tongue-flicks	May not occur or be reduced by biting (count post-bite tongue-flicks, increase sample size, use tongue-flick attack score if tongue-flicks occur regularly; use bites or bite latency if tongue-flicks do not occur or are rare)
		Number of individuals that bite	Low percentage of individuals may bite (increase sample size or use tongue-flick attack score)
		Latency to bite swab	Low percentage of individuals may bite (increase sample size or use tongue-flick attack score)
		Tongue-flick attack score	Ineffective if no tongue-flicking or biting (use other methods such as presentation on labeled substrates away from the investigator)
		Extrapolated tongue-flick rate	May be inflated for bites at short latency (use tongue-flick attack score or accept heavy weighting for short latency bites)
	Does not work for taxa that do not tongue-flick or bite swabs; does not apply to location of food by scent-trailing		
Predators	Quick, shows differential response in some taxa	Tongue-flicks	May be rare, especially in eublepharids (use displays or alternative methods such as study of avoidance or change of activity in response to labeled substrates; should be used to confirm even significant discrimination based on tongue-flicking alone)

TABLE 3. Continued.

Stimuli	Advantages and disadvantages	Variables	Possible limitations (solutions)
		Antipredatory displays	Absent in many taxa (use alternative methods)
	Does not work for taxa that do not tongue-flick swabs; often does not reveal independently convincing reactions to predators		
Pheromones	Often provides evidence of biologically important conspecific and interspecific discriminations	Tongue-flicks	May not occur (use displays or methods such as presentation on tile substrates, chemical transfer among individuals used as stimulus sources, or scent-trailing)
		Species-typical social displays, scent-marking	May not occur (use other methods)

A second way to deal with the problem is to extrapolate the tongue-flick rate from the portion of a trial prior to biting to obtain an expected number of tongue-flicks throughout the trial interval (Arnold, 1978; Halpern and Frumin, 1979; Cooper and Burghardt, 1990). A potential problem is that the extrapolation may be inaccurate, especially if bites occur in the first few seconds, because the tongue-flick rate is often highest early in trials. Nevertheless, the method performed well in a comparative analysis of scoring methods used with swab tests (Cooper and Burghardt, 1990).

Another approach is to continue to count tongue-flicks for the remainder of the test interval after the bitten swab has been released. In a study of food chemical discrimination by the iguanid lizard *Dipsosaurus dorsalis*, biting prevented detection of any obvious differences by the usual criterion of numbers of tongue-flicks prior to biting. However, the total number of tongue-flicks before and after biting was significantly greater in response to stimuli from several food sources than from controls (Cooper and Alberts, 1990). This method seems to warrant additional use.

As noted earlier, some lizards, especially geckos, may attack swabs prior to any tongue-flicking. When such biting has occurred in my studies, I have discarded data for the animal on the grounds that no tongue-flicking has occurred and the bite was based on unknown cues, possibly visual rather than chemical.

This has not been much of a problem in my comparative studies because in most taxa no individuals bit prior to tongue-flicking the swab.

A possible role of olfaction in triggering predatory attack should be considered when biting occurs without prior tongue-flicking and could be demonstrated by differential response to airborne chemicals in conjunction with selective sensory elimination experiments. Olfactory identification of prey cues is strongly suggested by the high frequency of attack without prior tongue-flicking by *Coleonyx variegatus* on swabs bearing prey chemicals, but not swabs bearing other stimuli (Dial et al., 1989). If tongue-flicking rarely occurs in response to prey chemical stimuli and a high percentage of attacks occurs without tongue-flicking, tongue-flicking is obviously not a useful variable for studying the response to prey chemicals. I have observed instances of biting without first tongue-flicking in a few taxa: one of 18 *Coleonyx variegatus* bit in response to prey chemicals, two of two *Phelsuma madagascariensis* bit in response to cricket chemicals and cologne, and some *Eumeces laticeps*, *Xantusia* vigilis, and *Tupinambis rufescens* bit in response to prey chemicals, cologne, and water (unpublished observations). A possible reason for the lower frequency of attack without tongue-flicking by *C. variegatus* in my laboratory than in Dial's is that my lizards were long-term captives with replete caudal fat stores.

Swab tests are useful for detecting the ability to identify prey chemicals in lizards that investigate stimuli lingually prior to attack. They may be suitable for species that do not, but tongue-flicking obviously cannot be used to detect the discrimination. They are not directly applicable to study of some important roles of chemical senses in feeding behavior. Location of hidden prey, which seems quite likely in species capable of prey chemical discrimination, must be demonstrated by other means. Swab tests are of very limited utility in studying strike-induced and postprandial chemosensory searching and related behaviors used to locate specific foods (Chiszar and Scudder, 1980; but see Cooper, 1992a).

Predators

Although tongue-flicking may provide useful information about the ability of squamates to respond differentially to chemical stimuli from predator and nonpredator species, the most convincing evidence for chemosensory response to predator cues from swab tests is performance of species-typical antipredatory displays. When exposed to chemical stimuli from the ophiophagous snake *Lampropeltis getulus*, many species of pit vipers adopt a defensive posture called body-bridging that is normally used only to ward off attack by *Lampropeltis* sp. (Weldon and Burghardt, 1979). Preliminary evidence suggests that naive racers (*Coluber constrictor mormon*) perform a jerky, spasmodic avoidance response to scorpion extracts (Burghardt, 1978).

Elicitation of specific antipredatory behaviors by chemical cues has also been reported in lizards. *Varanus albigularis* laterally compresses the body, hisses, and performs tail slapping and vent dragging (the latter a territorial behavior) in response to chemical stimuli from venomous snakes, but not in response to nonvenomous snakes; the lizards attack nonvenomous snake species, but not venomous ones (Phillips and Alberts, 1992). *Coleonyx variegatus*, a large majority of which did not tongue-flick prior to displaying, elevated their tails in response to chemical cues from a predatory snake (Dial et al., 1989; Dial, 1990). On the basis of these findings, Dial and Schwenk (1996) questioned the utility of cologne as a pungency control. I agree, regardless of the precise effects of cologne, because in studies including stimuli from nonpredatory species, a pungency control seems redundant. If cologne is used, it should be diluted well below the threshold of noxiousness, as discussed for studies of prey chemical discrimination, to avoid misleading defensive reactions. In a few trials with undiluted cologne, *C. variegatus* backed away from the swabs and either undulated their tails or elevated them slightly, suggesting a weak rendition of a full elevated tail display (Cooper, unpublished).

The swab method provides suggestive, but less compelling results when no obvious independent measure of antipredatory behavior occurs (Table 3). Some colubrid snakes and broad-headed skinks (*Eumeces laticeps*) tongue-flick at higher rates to skin chemicals from predatory snakes than from nonpredatory snakes (Weldon, 1982; Cooper, 1990a), but perform no overt defensive behaviors. In the latter study, tongue-flick attack scores were lower in response to all snake stimuli than to food stimuli, suggesting that the elevated tongue-flicking rate reflects identification of the predator stimuli as such rather than as potential food stimuli. However, the swab method is inadequate to assess effects of predator chemicals on species-typical antipredatory behavior in *E. laticeps*. For species lacking obvious displays, responses to substrates labeled with predator chemicals may be superior for studying avoidance and related responses. This approach has been used successfully to study response to predator chemicals in a lacertid lizard (Thoen et al., 1986).

Pheromones

Tongue-flicking rates in swab or tile experiments on responses to pheromones frequently have unambiguous interpretations (Table 3). For example, higher tongue-flick rates to chemical stimuli from either or both sexes of conspecifics than to controls indicates detection, whereas a higher rate to one sex when both are detected indicates differential response (Duvall, 1979; Cooper and Vitt, 1984a; Alberts, 1989). Similar tests can be used to relate responsiveness to the reproductive condition of stimulus sources and respondents, to species, to the chemical fraction of pheromones, etc. (reviewed by Halpern, 1992;

Mason, 1992). Composite variables analogous to the tongue-flick attack score can be constructed for pheromonal studies by combining tongue-flicking with species-typical social display (Steele and Cooper, 1997). Nevertheless, methods other than swab and other labeled-substrate tests must be used to detect some important responses to pheromones. Odor transfer between individuals has been used to detect pheromonal stimulation of courtship (Cooper et al., 1986) and aggression (Cooper and Vitt, 1987), and scent-trailing has been studied in mazes (Ford, 1982; Cooper and Vitt, 1986c). Another specific behavior useful for detecting response to pheromones is scent-marking in response to an individual's own pheromones (*Dipsosaurus dorsalis*) (Alberts, 1992).

EVALUATION

Swab tests of tongue-flicking and other responses provide rapid, conclusive assays of the ability of many squamates to respond differentially to biologically important stimuli. Their wide use in comparative studies of ecological, evolutionary, genetic, and experiential factors has led to much of our current understanding of squamate chemosensory roles. Although the method continues to be highly productive, it has several limitations. Results must be interpreted cautiously if no significant differences are detected among stimuli or treatments. Chemical stimuli must be invisible or visually identical to the animals being tested. Several things discussed above must be considered when designing and interpreting results. Among these are issues specific to studies of the particular categories of stimuli (food, predator, and pheromonal), including the types and utility of control substances for pungent and odorless stimuli, inferences to be made regarding senses mediating discriminations, and effects of experimenter presence and proximity.

In some cases, swab tests are not applicable. In others, methods other than swab tests and response measures other than tongue-flicking may be superior. It is important to recognize that swab tests reveal capacities for making chemical discriminations in the absence of other cues. Demonstration that a discrimination can be made does not permit inferences about the importance of the ability when cues in other sensory modalities are present. For example, a lizard that can discriminate prey chemicals from control substances in swab tests may rely primarily on visual cues to initiate attack when both chemical and visual cues are present. Attacks may be triggered by visual cues without prior detection of chemical cues (Burghardt, 1973; Nicoletto, 1985). One cannot conclude from the ability to make chemical discriminations in swab tests that animals perform the discriminations reliably under field conditions, but it may be inferred that they have the ability to do so. Despite these limitations, swab tests and related tests involving tongue-flicking remain by far the best approach for rapid collec-

tion of comparative data on squamate chemosensory abilities in a wide range of contexts.

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NOVEL ANTIFEEDANT AND INSECTICIDAL COMPOUNDS FROM AVOCADO IDIOBLAST CELL OIL

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Abstract—Several insecticidal compounds have been identified by bioassay-driven fractionation of avocado, *Persea americana* Mill, idioblast cell oil. A flash chromatography fraction of the oil showed substantial toxicity to early instars of the generalist insect herbivore, *Spodoptera exigua* (Hübner) (100% mortality after seven days). Following further fractionation, five biologically active compounds, 2-(pentadecyl)furan, 2-(heptadecyl)furan, 2-(1*E*-pentadecenyl)furan, 2-(8*Z*,11*Z*-heptadecadienyl)furan, and the triglyceride triolein, were identified. Several minor components were also tentatively identified, including 2-(1*Z*-pentadecenyl)furan, 2-(1*E*-heptadecenyl)furan, and 2-(1*E*,8*Z*,11*Z*-heptadecatrienyl)furan. Several 2-alkylfurans of this type have been reported previously from avocado (*Persea* spp.) and have received the common name of avocadofurans. The major compounds were tested individually for toxic and growth inhibitory effects. Individually, the compounds had low to moderate toxicity. Of these, 2-(pentadecyl)furan had the greatest effects, with an LC_{50} value of 1031 μg/g. At concentrations of 600 μg/g or higher in diets, larval growth was inhibited by >70% compared to controls. The analogous 2-(heptadecyl)furan had an LC_{50} value of 1206 μg/g, and also significantly reduced larval growth (>75% versus controls) at concentrations of >600 μg/g. The unsaturated analogs 2-(1*E*-pentadecenyl)furan and 2-(8*Z*,11*Z*-heptadecadienyl)furan were less toxic. Triolein was only weakly toxic, with an LC_{50} value of 10,364 μg/g diet. Larval growth was inhibited only at concentrations of 7000 μg/g or higher. The potential of avocadofurans in insect control is discussed.

Key Words—*Spodoptera exigua*, *Persea americana*, avocado, idioblast cells, avocadofuran, 2-(pentadecyl)furan, 2-(heptadecyl)furan, triolein.

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INTRODUCTION

Avocados, *Persea americana* Mill (Lauraceae), are oleaginous fruit in which oil levels in the mesocarp (or flesh) vary from 1–2% (fresh weight) early in the season to over 30% late in the season (Biale and Young, 1971). The oils are valuable nutritionally as a source of energy, vitamins, and unsaturated lipids, with the edible portion of the fruit being rich in oleic, palmitic, linoleic, and palmitoleic acids (Biale and Young, 1971). Avocado oils also are used extensively in cosmetics (Anonymous, 1980).

Compounds isolated from avocado fruit also have been reported to have various types of biological activity. For example, 1-acetoxy-2-hydroxy-4-oxoheneicosa-(12*Z*,15*Z*)-diene (persin) extacted from fresh avocado leaves induced vomiting and inhibited growth of fourth-instar *Bombyx mori* L. (Chang et al., 1975; Murakoshi et al., 1976) at a concentration of 200 μg/g within two days. Prusky et al. (1982) subsequently isolated persin from peels of unripe avocado fruit and characterized its antifungal activity against *Colletotrichum gloeosporioides* Penz. In addition, Prusky et al. (1991) found that a related compound, 1-acetoxy-2,4-dihydroxy-*n*-heptadec-16-ene, isolated from the peel and flesh of unripe avocado fruit, also was active as a fungicide. A further study identified a series of related compounds with antifungal activity, including 1,2,4-trihydroxyheptadec-16-yne, 1,2,4-trihydroxy-*n*-heptadec-16-ene, and 1-acetoxy-2,4-dihydroxyheptadec-16-yne (Adikaram et al., 1992).

All aforementioned compounds were isolated from crude homogenates of plant tissues rather than from specific cells or structures. In contrast, our work has focused on chemicals present in a specific cell type. Idioblast cells are specialized cells that markedly differ from other constituents of the same tissue in form, structure, and contents (Esau, 1967). Avocado leaves, seeds, roots (Armstrong, 1964), and fruit (Platt-Aloia et al., 1983; Platt-Aloia and Thomson, 1992) contain specialized idioblast oil cells scattered throughout the avocado mesocarp, composing approximately 2% of the tissue volume (Cummings and Schroeder, 1942). Moreover, the cells have been reported to contain an oil that differs from other lipids found in the fruit mesocarp (Platt and Thomson, 1992).

In recent studies, Kobiler et al. (1993) demonstrated the antifungal activity of two compounds present in these idioblast oil cells to the fungus, *C. gloeosporioides*. Rodriguez-Saona and Trumble (1996) found that the crude oil extracted from idioblast cells was not only toxic to early (LC_{50} = 1600 μg/g) and late instars (LC_{50} = 5100 μg/g) of the herbivore, *Spodoptera exigua* (Hübner), but also deterred feeding. Rodriguez-Saona et al. (1997) subsequently isolated and identified persin from idioblast cell oil by bioassay-driven fractionation and demonstrated its detrimental effects to *S. exigua* larvae. Persin inhibited larval growth when mixed with artificial diet at concentrations of 200 μg/

g. At concentrations of 400 μg/g or above, persin significantly deterred feeding and reduced larval weight by more than 70%.

A second, less polar fraction from the initial flash chromatography fractionation of the idioblast cell oil was also toxic to *S. exigua* larvae. The objectives of the present study were: (1) to identify the biologically active compound(s) in this fraction, (2) to synthesize the active compound(s), and (3) to test the synthetic compound(s) for mortality and growth effects on *S. exigua*.

METHODS AND MATERIALS

Extraction of Idioblast Cell Oil

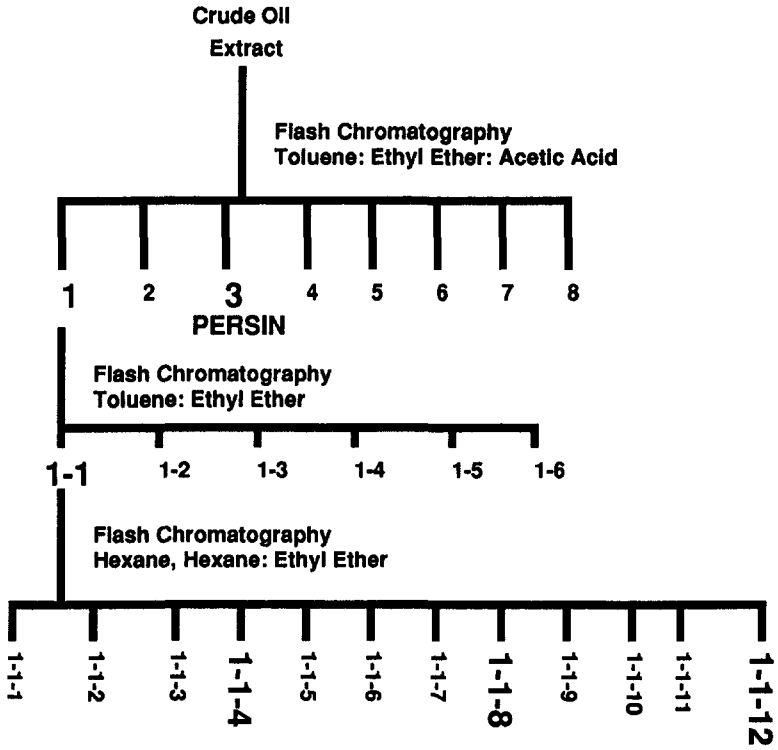
Hass avocado fruit were collected from trees at the South Coast Research and Extension Center, University of California, Santa Ana, California. Idioblast cells were separated from ripe fruit and the oil was extracted as previously described (Rodriguez-Saona and Trumble, 1996).

Insects. S. exigua larvae were used in all experiments. Larvae were reared on artificial diet modified from Patana (1969), and maintained at 28 $\pm$ 2°C and 14L:10D photoperiod. The colony was originally collected from Orange County, California, and had new material added within 12 month prior to the study. The age of the cohorts tested was standardized by using neonates within 12 hr of eclosion. All bioassays were maintained at 28 $\pm$ 2°C, 75% relative humidity, and 14L:10D photoperiod with fluorescent lighting.

Isolation and Identification of Active Compounds

Flash chromatography was carried out with 230–400 mesh silica gel (Aldrich, Milwaukee, Wisconsin). Electron impact (70 eV) mass spectra of volatile compounds were taken on a Hewlett-Packard 5890 GC interfaced to a 5970 mass selective detector (carrier gas, helium; head pressure, 105 kPa). A DB5-MS column was used (30 m $\times$ 0.2 mm ID, J&W Scientific, Folsom, California). Chemical ionization (methane) mass spectra were obtained with a 5890 GC (DB5 column, 30 m $\times$ 0.25 mm ID) interfaced to an HP 5989A mass spectrometer. Fast atom bombardment (FAB) spectra were recorded with a VG ZAB-2fHf instrument (VG Instruments, Danvers, Massachusetts), and high resolution exact mass spectra were taken on a VG 7070E double-focusing magnetic sector instrument. ^{1}H NMR spectra were recorded with a QE-300 instrument (General Electric, Fremont, California) in $CDCl_3$.

The crude idioblast cell oil was fractionated as shown in Scheme 1. The initial steps of the fractionation have been previously described (Rodriguez-Saona et al., 1997). The lowest polarity fraction from the initial flash chromat-



Scheme 1.

ographic separation (fraction 1, 3.67 g total weight) was further purified by flash chromatography (5 cm ID × 25 cm column). The column was eluted sequentially with 2 liters each of toluene–ethyl ether 95:5 and 90:10 (v/v). Material remaining on the column was stripped off with ethanol (2 liters). Fractions were checked by thin layer chromatography (TLC) on silica plates developed with toluene–ethyl ether (90:10, v/v). Spots on developed plates were visualized under UV light (254 nm), followed by spraying with H_2SO_4 and charring with a heat gun. Subfractions were combined to yield 6 fractions (1–1 to 1–6), which were concentrated under reduced pressure and then pumped under vacuum (~0.5 mm Hg) to remove traces of solvent. The concentrated fractions were weighed, diluted with acetone to a final volume of 10 ml, and refrigerated at 4°C until bioassayed.

A portion (~75%) of the most active fraction (frac. 1–1, 1.3 g total weight) was fractionated further by flash chromatography (5 cm ID × 25 cm). The

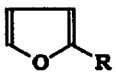
elution solvents used sequentially, were 2 liters of hexane, 1 liter of hexane–ethyl ether (95:5, v/v), 0.4 liter of hexane–ethyl ether (90:10), and 0.4 liter of ethyl ether. Twelve fractions were collected, concentrated, and tested for activity. Subfractions 1-1-4 (0.08 g), 1-1-8 (0.09 g), and 1-1-12 (0.89 g) contained most of the mass of material (6, 7, and 68%, respectively) and were most active in bioassays. Structures of identified compounds are shown in Figure 1.

Fraction 1-1-4

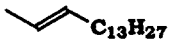
Compound 1 (39.2%). MS (70 eV): 278 (17), 249 (2), 235 (5), 221 (2), 207 (2), 193 (2), 179 (3), 165 (3), 151 (10), 137 (10), 123 (20), 95 (57), 82 (44), 81 (100). CI-MS (methane): 277 (100, M-1), 279 (98, M + 1), 307 (25; M + 29).

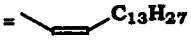
Compound 2 (49.4%). MS (70 eV): 276 (25), 247 (1), 233 (1), 219 (2), 191 (1), 177 (2), 163 (3), 149 (8), 135 (12), 121 (9), 107 (61), 94 (100), 81 (19), 79 (24), 77 (19). CI-MS (methane): 275 (61, M-1), 277 (100, M + 1), 305 (25, M+ 29).

Compound 3 (2.8%). MS (70 eV): 276 (35), 247 (2), 233 (2), 219 (3), 191 (3), 177 (2), 163 (4), 149 (9), 135 (15), 121 (11), 107 (60), 94 (100), 81

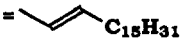


1: R = $C_{15}H_{31}$

2: R = 

3: R = 

4: R = $C_{17}H_{35}$

5: R = 

6: R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

7: R = $CH{=}CH(CH_2)_5CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

8: R' = $C(O)(CH_2)_7CH{=}CH(CH_2)_7CH_3$

FIG 1. Structures of compounds identified from avocado idioblast cell oil fractions.

(17), 79 (21), 77 (20). CI-MS (methane): 275 (62, M-1), 277 (100, M + 1), 305 (24; M + 29).

Compound 4 (2.6%). MS (70 Ev): 306 (28), 263 (5), 249 (4), 207 (6), 179 (3), 165 (3), 151 (13), 137 (17), 123 (35), 95 (63), 82 (54), 81 (100). CI-MS (methane): 305 (100, M-1), 307 (82, M + 1), 335 (18, M + 29).

Compound 5 (6.0%). MS (70 eV): 304 (31), 247 (2), 219 (1), 193 (1), 177 (3), 163 (4), 149 (11), 135 (16), 121 (9), 107 (48), 95 (20), 94 (100), 81 (20), 79 (20). CI-MS (methane): 303 (65, M-1), 305 (100, M + 1), 333 (19).

^1H NMR of Fraction 1-1-4 ($CDCl_3$). δ 0.88 (ragged t, $J \sim 6.9$ Hz), 1.2–1.4 (m), 1.4–1.8 (m), 2.17 (m), 2.61 (t, $J = 7.5$ Hz), 5.96 (d, $J = 1.4$ Hz), 6.13 (d, $J = 1.6$ Hz), 6.18 (t, $J = 1.8$ Hz), 6.27 (distorted dd), 6.33 (distorted dd), 7.29 (m).

Fraction 1-1-8.

Compound 6 (83%). MS (70 eV): 302 (14), 273 (5), 259 (5), 245 (8), 231 (7), 217 (5), 203 (3), 189 (3), 175 (3), 161 (4), 149 (6), 135 (9), 121 (13), 107 (12), 95 (34), 94 (36), 81 (100), 67 (47), 55 (30), 41 (48). HR-MS: Calcd for $C_{21}H_{34}O$: 302.2610; found: 302.2599. ^1H NMR: δ 0.89 (3H, t, $J = 7.7$ Hz, CH_3), 1.25–1.5 (14H, m, 7 methylenes), 1.63 (2H, m, $H_{2'}$), 2.05 (4H, m, $H_{7'}$, $H_{13'}$), 2.61 (2H, t, $J = 7.6$ Hz, $H_{1'}$), 2.78 (2H, br. t, $J = 6$ Hz, $H_{10'}$), 5.37 (4H, m, $H_{8',9',11',12'}$), 5.97 (1H, d, $J = 1.3$ Hz, H_3), 6.28 (1H, m, H_4), 7.29 (1H, distorted d, H_5).

Compound 7 (17%). MS (70 eV): 300 (32), 271 (3), 257 (3), 243 (7), 229 (20), 215 (6), 204 (5), 201 (5), 191 (9), 175 (11), 161 (9), 147 (15), 133 (21), 121 (27), 120 (31), 107 (73), 94 (100), 81 (71), 79 (86), 77 (63), 67 (52), 55 (43), 41 (65). HRMS: Calcd for $C_{21}H_{32}O$: 300.2453; found: 300.2451. Diagnostic ^1H NMR peaks (from the spectrum of the mixed major and minor compounds): δ 2.18 (2H, br. quart), 6.12 (1H, m), 6.18 (1H, m), 6.34 (1H, br d). Other NMR signals for this compound were obscured under the signals from the major component of this fraction.

Fraction 1-1-12

Reduction with $LiAlH_4$. Two milligrams of the fraction were stirred with 10 mg of $LiAlH_4$ in ether (1 ml) at room temperature for 2 hr. The mixture was cautiously quenched with 1 M aq. HCl and extracted with ether. The dried (Na_2SO_4) extract was analyzed by GC-MS (DB5-MS column, 20 m × 0.2 mm ID, temperature program 50°C for 1 min then 10°/min to 250°C). The retention times and mass spectra of the sample components were compared with those of authentic samples of hexadecyl (palmityl) and *Z*9-octadecenyl (oleyl) alcohols. The mass spectrum and retention time of the third component [tentatively identified as *Z*9-hexadecenyl (palmitoleyl) alcohol] were compared with those of an

authentic standard of Z11-hexadecenyl alcohol, providing close but not exact matches (retention times different by 0.05 min).

Base Hydrolysis. Two milligrams of the fraction were dissolved in 0.5 ml EtOH, and 1 drop of 20% aq. NaOH was added. The mixture was stirred at room temperature 2 hr, then acidified with 1 M HCl and extracted with ether. The dried (Na_2SO_4) extract was analyzed by GC (DB-5, 30 m × 0.32 mm ID, temperature program 50°C for 1 min, 15°/min to 275°C), and the retention times of the sample components were compared with those of authentic standards of oleic, palmitic, and palmitoleic acids.

^{1}H NMR ($CDCl_3$): δ 0.88 (9H, distorted t), 1.2–1.4 (m), 1.61 (~6H, m), 2.02 (12H, m), 2.32 (6H, distorted t, J ~ 7.4 Hz), 4.15 (2H, dd, J = 11.9, 6 Hz), 4.30 (2H, dd, J = 11.9, 4 Hz), 5.27 (1H, m), 5.33 (6H, m). FAB-MS (nitrobenzyl alcohol matrix): highest mass peak cluster centered at m/z 603 ($M-C_{18}H_{35}O_2$).

Bioassays

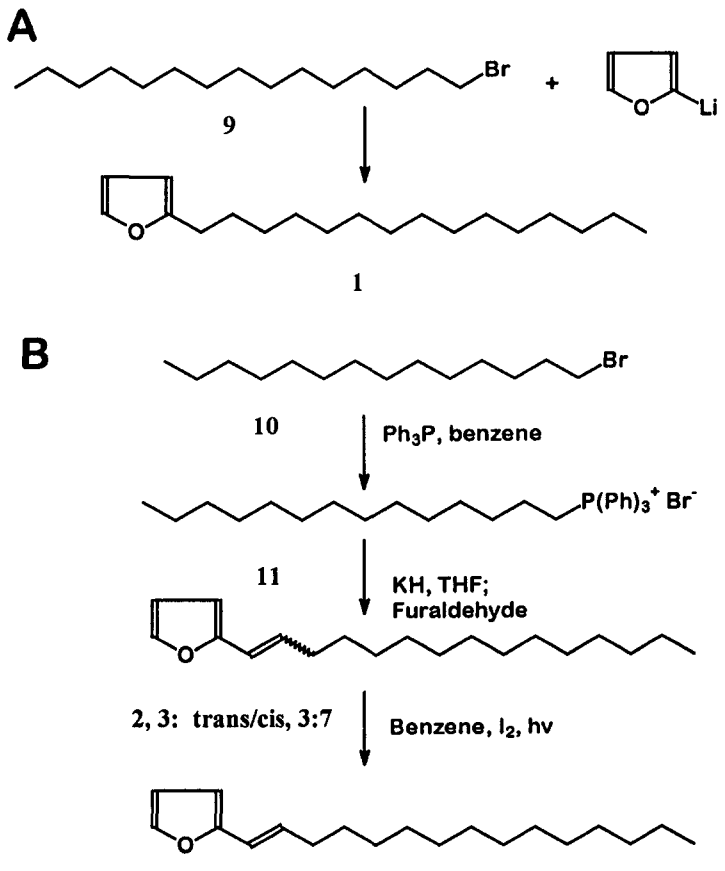
The insecticidal activity of the fractions was tested with artificial diet bioassays. Treated diets were prepared by transferring 750 μl of acetone solutions of each fraction (equivalent to 300 mg crude idioblast cell oil) into 50-ml polypropylene centrifuge tubes (Fisher, Pittsburgh, Pennsylvania), evaporating the acetone, adding 2 ml of 0.1% Tween 80 solution (Fisher), homogenizing with an ultrasonic homogenizer (Cole-Parmer, Chicago, Illinois), and adding artificial diet to produce a final weight of 15 g containing the equivalent of 2% idioblast oil. The mixture was vortexed for 3 min. Control diet was prepared by mixing 2 ml of Tween solution and 13 g of artifical diet to produce a final weight of 15 g. Control and treated diets were poured into 16-well (15.9-mm-diameter and 15.9-mm-deep) bioassay trays (C-D International Inc., Pitman, New Jersey). One neonate was added per well, and trays were placed in an incubator under the previously described conditions. Twenty-four neonates were tested for each fraction and control. Mortality and larval weights were recorded after seven days.

Synthesis of Furan Compounds

Reactions were carried out under a N_2 atmosphere unless otherwise stated. Flash chromatography was carried out with 230–400 mesh silica gel. THF was dried by distillation from sodium-benzophenone ketyl. ^{1}H NMR spectra were obtained on a 270-MHz JEOL NMR spectrometer, in $CDCl_3$ or C_6D_6. ^{13}C NMR spectra were obtained at 67.9 MHz in $CDCl_3$. Infrared spectra were obtained on a Mattson Galaxy 2000 FT-IR with NaCl plates or in carbon tetrachloride solution. Mass spectra (HR-MS and LR-MS) were obtained from the University of California Riverside, Mass Spectroscopy Laboratory.

2-(Pentadecyl)furan (1) (Scheme 2A)

A dry 50-ml round-bottomed flask charged with furan (1.40 g, 20.6 mmol) and THF (10 ml) was cooled to −78°C and *n*-butyllithium (13.6 ml, 1.50 M in hexanes, 20.6 mmol) was added dropwise. The solution was stirred for 30 min at −78°C, then warmed to 0°C and placed in an ice bath for 1 hr. The mixture was then recooled to −78°C and 1-bromopentadecane **9** (5 g, 17.2 mmol) in THF (10 ml) was added dropwise. The resulting solution was stirred for 1 hr, warmed to room temperature, and stirred for 12 hr. The reaction was



Scheme 2.

quenched with a saturated solution of NH_4Cl (5 ml). The organic layer was separated and the aqueous layer extracted with ether (3 × 20 ml). The combined organic layers were washed with saturated $NaHCO_3$ (1 × 20 ml), brine (1 × 20 ml), dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography on silica, eluting with hexanes to afford an oily liquid that was recrystallized from methanol (~50 ml/g, cooling to 5°C) to afford 4.8 g (64%) of 2-(pentadecyl)furan (**1**). ^1H NMR: ($CDCl_3$) δ 0.85 (3H, distorted t, J = 7.2 Hz, CH_3), 1.1–1.4 (24H, broad m, 12 methylenes), 1.57 (2H, quintet, J = 7.2 Hz, $H_{2'}$), 2.58 (2H, t, J = 7.2 Hz, $H_{1'}$), 5.99 (1H, dd, J = 1, 3.0 Hz, H_3), 6.29 (1H, dd, J = 1.8, 3.0 Hz, H_4), 7.29 (1H, dd, J = 1, 1.8 Hz, H_5).

2-(Heptadecyl)furan (4)

1-Heptadecanol (10 g, 39.1 mmol) was placed in a dry 25-ml round-bottomed flask attached to a reflux condenser and heated to 60°C under a nitrogen atmosphere. Phosphorous tribromide (10 g, 39.1 mmol) was added dropwise and the resulting solution was stirred for 48 hr. The reaction mixture was then cooled in an ice bath and quenched with a saturated solution of $NaHCO_3$ (10 ml). The aqueous layer was extracted with ether (4 × 10 ml). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The crude residue was flash chromatographed (hexanes) to afford 7.2 g (57%) of 1-bromoheptadecane as a colorless oil. ^1H NMR ($CDCl_3$): δ 0.85 (3H, t, J = 6.5 Hz), 1.1–1.4 (28H, broad s), 1.86 (2H, quintet, J = 6.8 Hz), 3.41 (2H, t, J = 6.8 Hz). 2-(Heptadecyl)furan **4** was prepared exactly as described above for 2-(pentadecyl)furan, by substituting 1-bromoheptadecane for 1-bromopentadecane. The chromatographed product was recrystallized from methanol as described above to afford 2.5g of 2-(heptadecyl)furan **4** in 48% yield. ^1H NMR ($CDCl_3$): δ 0.86 (3H, distorted t, J = 7.2 Hz, CH_3), 1.1–1.4 (28H, broad m, 14 methylenes), 1.60 (2H, quintet, J = 7.2 Hz, $H_{2'}$), 2.58 (2H, t, J = 7.2 Hz, $H_{1'}$), 5.95 (1H, dd, J = 1.0, 2.7 Hz, H_3), 6.26 (1H, J = 1.8, 2.7 Hz, H_4), 7.30 (1H, dd, J = 1.0, 1.8 Hz, H_5).

2-(1Z-pentadecyl)furan (3) (Scheme 2B)

A mixture of 1-bromotetradecane **10** (10.0 g, 36.1 mmol), triphenylphosphine (10.5 g, 36.1 mmol), and benzene (25 ml) was refluxed for 48 hr, and the mixture was concentrated under reduced pressure to a viscous oil. Addition of ether afforded a white solid, which was dried under vacuum for 24 hr at 65°C, yielding 18.9 g (92%) of tetradecyltriphenylphosphonium bromide **11**. It was used without further purification.

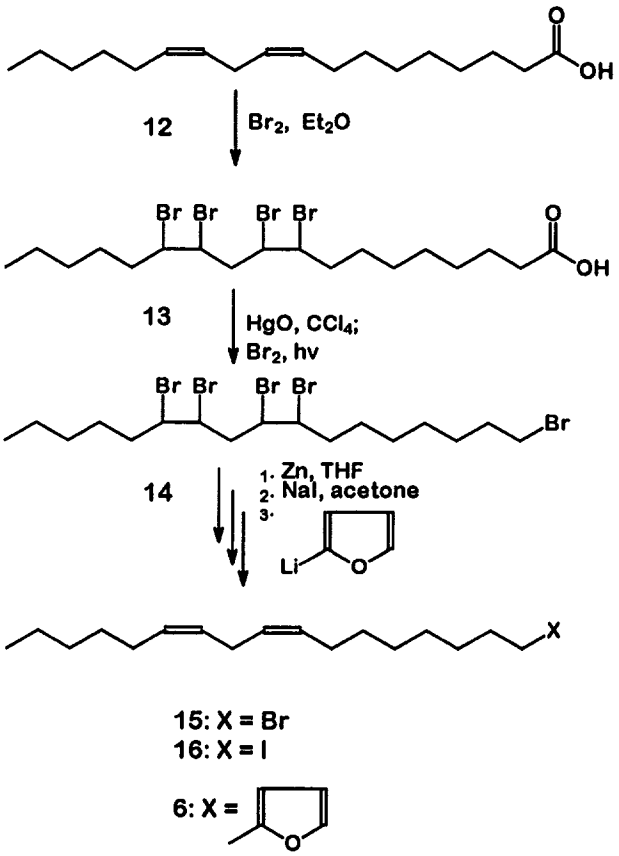
Potassium hydride (2.33 g, 20.2 mmol, 35% oil dispersion) was washed with hexanes (3 × 5 ml) and pumped under vacuum. After 5 min, the flask was filled with N_2, dry THF (50 ml) was added, and the reaction flask cooled in an ice bath. The phosphonium salt **11** (10 g, 18.5 mmol) was added in small portions over a 20-min period. After stirring for 15 min, the resulting yellow solution was warmed to room temperature, stirred for 30 min, and then cooled to −78°C. Freshly distilled furaldehyde (2.1 g, 22.2 mmol) in THF (25 ml) was added dropwise over 15 min. The resulting solution was stirred for 1 hr, warmed to room temperature, and stirred 18 hr. The reaction was quenched with saturated aq. NH_4Cl (50 ml). The organic layer was separated and the aqueous layer was extracted with ether (3 × 20 ml). The combined organic layers were washed with saturated $NaHCO_3$ (1 × 25 ml) and brine (1 × 25 ml), dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography (hexanes), to afford 4.2 g (81%) of a mixture of furans **3** and **2** (7:3 *cis/trans* ratio). ^1H NMR of major product: ($CDCl_3$): δ 0.92 (3H, distorted t, J = 7.0 Hz, CH_3), 1.2–1.6 (22H, m, 11 methylenes), 2.45 (2H, apparent dq, J = 1.8 Hz, 7.1 Hz, $H_{3'}$), 5.58 (1H, dt, J = 11.6, 7.1 Hz, $H_{2'}$), 6.18 (1H, dt, J = 7.1, 1.8 Hz, $H_{1'}$), 6.24 (1H, d, J = 3.4 Hz, H_3), 6.40 (1H, dd, J = 3.2, 2.0 Hz, H_4), 7.38 (1H, d, J = 1.7 Hz, H_5).

2-(1E-pentadecenyl)furan(2) (Scheme 2B)

The diastereomeric mixture of 2-(1-pentadecenyl)furans **2** and **3** (1.0 g, 3.4 mmol) was placed in a stirred 2% iodine/benzene solution (10 ml) in an open beaker and irradiated with a fluorescent light. The *cis* to *trans* isomerization was monitored by NMR [scanned unlocked, observing the disappearance of the resonance at δ = 5.58 (1H, dt, J = 11.9 Hz, 6.9 Hz, *cis* isomer) and appearance of the resonance at δ = 6.3 (1H, dt, J = 16.1 Hz, 6.9 Hz, *trans* isomer)]. After ~30 min of irradiation, the reaction mixture was washed thoroughly with saturated sodium thiosulfate. The organic layer was separated, dried over $MgSO_4$, filtered, and concentrated. The crude product was passed through a silica gel plug and eluted with hexanes. The eluate was concentrated, and the oily residue was recrystallized from methanol as described above, filtering cold to afford 0.53 g (57%) of **2** as low-melting white crystals. ^1H NMR (C_5D_5): δ 0.90 (3H, distorted t, J = 7.0 Hz, CH_3), 1.2–1.5 (22H, m, 11 methylenes), 2.05 (2H, apparent q, J = 6.9 Hz, $H_{2'}$), 5.95 (1H, d, J = 3.2 Hz, H_3), 6.16 (1H, m, H_4), 6.20 (1H, d, J = 16.1 Hz, $H_{1'}$), 6.30 (1H, dt, J = 16.1 Hz, 6.9 Hz, $H_{2'}$), 7.06 (1H, s, H_5).

*2-(8Z,11Z-Heptadecadienyl)furan **6** (Scheme 3)*

9,10,12,13-Tetrabromooctadecanoic Acid (13). Bromine (10.6 g, 66.6 mmol) was added dropwise to a rapidly stirred solution of linoleic acid **12** (8.9



Scheme 3.

g, 31.7 mmol) in diethyl ether (264 ml) at 0°C. After stirring for 30 min, the solution was warmed to room temperature and quenched by addition of saturated aq. sodium thiosulfate (20 ml). After stirring for 10 min, the organic layer was separated and the aqueous layer extracted with ether (3 × 20 ml). The combined organic layers were washed with saturated aq. NH_4Cl (2 × 10 ml) and brine (1 × 10 ml), dried over $MgSO_4$, filtered, and concentrated. The crude product was recrystallized from pentane–ether (1 : 1) to afford 16.6 g (85%) of the tetrabromo acid **13** as white crystals. ^1H NMR ($CDCl_3$): δ 0.89 (3H, distorted t, J = 6.3 Hz), 1.1–2.1 (18H, m), 1.60 (2H, m), 1.83 (2H, m), 2.01 (2H, m), 2.34 (2H, t, J = 7 Hz), 2.48 (2H, m).

*1,8,9,11,12-Pentabromoheptadecane (**14**).* A 500 ml roundbottom flask, equipped with a reflux condenser and addition funnel, was charged with tetrabromo acid **13** (16.4 g, 27.3 mmol), carbon tetrachloride (135 ml), and mercuric

oxide (8.87 g, 41.0 mmol) and brought to a gentle reflux. Bromine (3.1 g, 41 mmol) was added dropwise over 30 min. After refluxing for 2 hr, the reaction was cooled to room temperature and quenched with a 10% solution of sodium thiosulfate (50 ml). The organic layer was removed and the aqueous layer was extracted with carbon tetrachloride (2 × 20 ml). The combined organic layers were washed with saturated aq. NH_4Cl (2 × 20 ml) and brine (1 × 20 ml), dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by flash chromatography (hexanes) and recrystallized (pentane) at 5°C to afford 10.9 g (63%) of pentabromide **14** as white crystals (mp, 62–64°C). ^1H NMR ($CDCl_3$): δ 0.88 (3H, distorted t, J = 6.5 Hz), 1.1–2.1 (18H, m), 2.50 (2H, m), 3.34 (2H, t, J = 7.3 Hz), 4.0–4.2 (2H, m), 4.32 (2H, m), 4.54 (2H, m).

(8Z,11Z)-1-Bromoheptadecadiene (15). Pentabromide **14** (5.35 g, 8.42 mmol) and zinc powder (1.2 g, 18.5 mmol) in THF (55 ml) were refluxed for 4 hr. The reaction mixture was then cooled to room temperature and concentrated under reduced pressure. The oily residue was extracted into ether–pentane (1:1), the solution was concentrated, and the crude product was purified by flash chromatography (pentanes) to afford 2.6 g (>95%) of the (*Z,Z*)-diene **15** as a colorless oil. ^1H NMR ($CDCl_3$): δ 0.87 (3H, distorted t, J = 6.9 Hz), 1.2–1.4 (14H, m), 1.82 (2H, quintet, J = 6.9 Hz), 2.03 (4H, apparent q, J = 6.8 Hz), 2.75 (2H, t, J = 5.9 Hz), 3.38 (2H, t, J = 7 Hz), 5.2–5.5 (4H, m).

(8Z,11Z)-1-Iodoheptadecadiene (16). (*Z,Z*)-Bromodiene **15** (2.67 g, 8.45 mmol) and sodium iodide (3.80 g, 25.3 mmol) in acetone (133 ml) were refluxed 4 hr. The reaction mixture was then cooled to room temp, filtered, concentrated, and extracted with ether (3 × 20 ml). The ether extracts were filtered concentrated, and the residue purified by flash chromatography (pentane) to afford 2.25 g (74%) of the (*Z,Z*)-iododiene **16**. ^1H NMR ($CDCl_3$): δ 0.87 (3H, distorted t, J = 6.9 Hz), 1.2–1.4 (14H, m), 1.79 (2H, quintet, J = 6.9 Hz), 2.03 (4H, apparent q, J = 6.9 Hz), 2.76 (2H, t, J = 5.9 Hz), 3.17 (2H, t, J = 7 Hz), 5.2–5.5 (4H, m).

2-(8Z,11Z-Heptadecadienyl)furan (6). Furan (1.1 g, 16.1 mmol) in THF (5 ml) was cooled to −78°C and *n*-butyllithium (5.4 ml, 1.5 M in hexanes, 8.1 mmol) was added dropwise. The resulting solution was stirred 30 min at −78°C, warmed to 0°C for 1 hr, and then recooled to −78°C. (*Z,Z*)-Iododiene **16** (2.94 g, 8.1 mmol) in THF (5 ml) was added via cannula, and the mixture was stirred 2 hr. The reaction was warmed to room temperature and stirred an additional 2 hr, and quenched with saturated aq. NH_4Cl (10 ml). The aqueous layer was separated and extracted with ether (3 × 10 ml). The combined organic layers were washed with saturated $NaHCO_3$ (2 × 10 ml) and brine (1 × 10 ml), dried over $MgSO_4$, filtered, and concentrated. The crude residue was passed through a silica gel plug, eluted with hexane, and then purified by HPLC (Rainin Dynamax column, 2.24 × 25 cm, Rainin Instruments, Emeryville, California) to afford 1.0 g (41%) of furanyl diene **6** as a colorless oil. ^1H NMR ($CDCl_3$):

δ 0.87 (3H, distorted t, J = 7.0 Hz), 1.2–1.4 (14H, m), 1.66 (2H, quintet, J = 6.6 Hz), 2.08 (4H, apparent q, J = 6.4 Hz), 2.64 (2H, t, J = 7.4 Hz), 2.80 (2H, t, J = 5.6 Hz), 5.2–5.5 (4H, m), 5.99 (1H, m), 6.28 (1H, m), 7.30 (1H, m).

Toxicity Studies

The effects of the synthetically produced avocadofuran compounds (>95% purity) and commercially available triolein (Sigma, St. Louis, Missouri) were tested on larval growth and mortality of *S. exigua*. Treated and control diet were prepared as previously described. Preliminary data (see results) showed that of the two compounds identified from subfraction 1-1-4, 2-(1*E*-pentadecenyl)furan **2** had little effect on survivorship and growth of *S. exigua* as compared to the saturated analog, 2-(pentadecyl)furan **1**. Similarly, 2-(8*Z*,11*Z*-heptadecadienyl)furan **6** was of much lower toxicity than 2-(heptadecyl)furan **4**. Thus, we focused our attention on the two saturated compounds. Six different concentrations (0, 600, 750, 900, 1050, and 1200 μg/g) were chosen on the basis of studies with the oil fractions and evaluated for both 2-(pentadecyl)furan and 2-(heptadecyl)furan. Triolein (subfraction 1-1-12) also was tested at 0 (control), 7000, 8000, 9000, 10,000, and 11,000 μg/g. Twenty-four neonates were used per treatment. Bioassays were conducted as previously described (Rodriguez-Saona et al., 1997) and replicated four times for each concentration (i.e., a total of 96 larvae was tested for each concentration). Larval weight, instar, and mortality were recorded after 7 days.

Data Analysis

Effective concentration (EC) values were calculated by subtracting the mean larval weight for each treatment from the mean weight of the controls, and dividing by the mean weight of the controls. Lethal concentration (LC) values were obtained from probit analysis (Finney, 1971). Growth index (GI) and Relative growth index (RGI) values were calculated as described by Zhang et al. (1993), but with an i_{max} of 3 (the stage attained by most control larvae after seven days). Statistical comparisons were conducted with SuperAnova (1989).

RESULTS

Isolation and Identification of Active Compounds

The least polar fraction from the first two flash chromatographic steps (fractions 1-1, Scheme 1) produced 100% larval mortality (Figure 2). In contrast, larvae fed on diet augmented with fractions 1-3 and 1-4 were significantly larger than control larvae (Figure 2B, $P < 0.05$). Further fractionation (Scheme

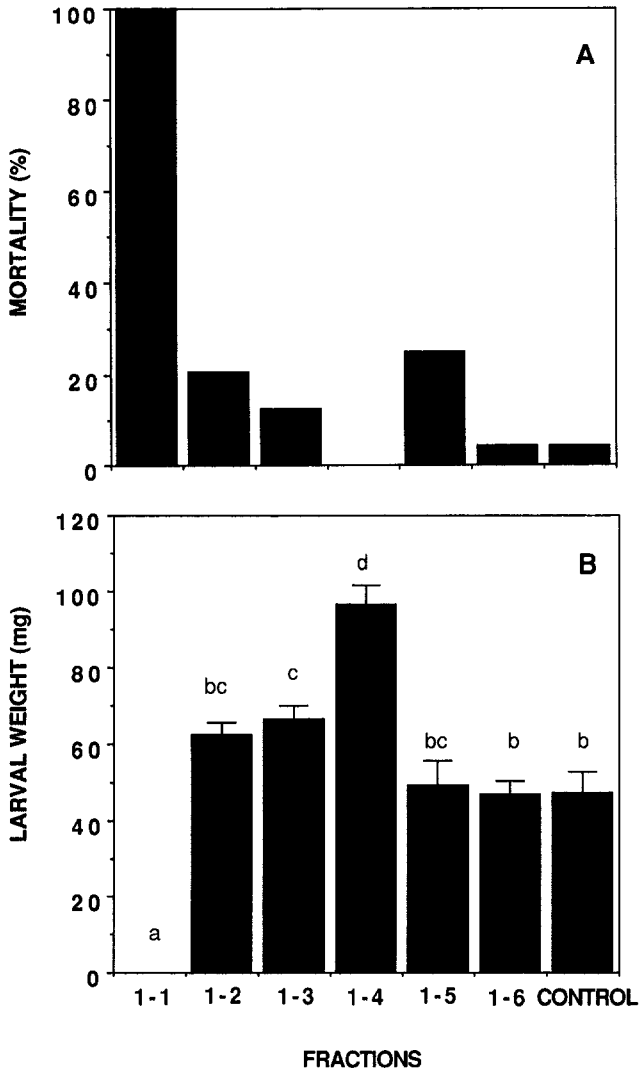


FIG 2. Percent mortality (A) and weight (B) of *S. exigua* larvae fed control diet and diet containing flash chromatography fractions of avocado idioblast cell oil. Different letters indicate statistical differences between treatments (Tukey's pairwise comparisons, $P < 0.05$). Fractions were bioassayed at concentrations equivalent to 20 mg crude oil per ml artificial diet.

1) of fraction 1-1 yielded three active subfractions (1-1-4, 1-1-8, and 1-1-12; Figure 3). These subfractions each significantly reduced larval weight compared to control larvae in feeding bioassays (Figure 3B, $P < 0.05$), although larval mortality was <30% (Figure 3A).

The least polar fraction, 1-1-4, consisted of two major and three minor components. The first major component gave a significant molecular ion (*m/z* 278, 17%), corresponding to a possible molecular formula of $C_{19}H_{34}O$, with three sites of unsaturation. The assignment of the molecular ion was confirmed by methane CI-MS, which gave large ions at *m/z* 277 and 279, and an M + 29 ion at *m/z* 307. The base peak in the EI spectrum (*m/z* 81) and a large fragment at *m/z* 95 (57%) were diagnostic for the monosubstituted 2-alkyl furans known to occur in avocado extracts (e.g., Weyerstahl et al., 1993). Furthermore, the entire series of ions from *m/z* 81 to 249, each separated by 14 mass units, was clearly visible, indicative of a saturated alkyl side chain. The furan ring accounted for all three sites of unsaturation, corroborating that the C-15 alkyl side chain was fully saturated. The ^{1}H NMR spectrum of the fraction showed peaks typical of a furan ring with a single alkyl substituent in position 2 (e.g., Kashman et al., 1969a,b), and no signals from methine protons or doublets from methyl branches, indicating that the alkyl chain was unbranched. Thus, this component was identified as 2-(pentadecyl)furan **1** (Figure 1), previously tentatively identified from avocado on the basis of MS data (Weyerstahl et al., 1993). The identification was confirmed by synthesis.

The second major component of this fraction gave a molecular ion at *m/z* 276, confirmed by methane CI-MS, suggesting it was an unsaturated analog of compound 1. The EI-MS showed a base peak at *m/z* 94 and a large fragment at *m/z* 107 (61%), diagnostic for a 2-substituted furan conjugated with a C=C double bond (Weyerstahl et al., 1993), and a series of ions, each separated by 14 mass units, from *m/z* 107 to 247, indicative of a saturated alkyl chain. The presence of the 1,2-disubstituted olefin conjugated to the furan ring and its geometry (*E*) were confirmed by comparison of multiplets at δ 6.13, 6.18, and 6.35 in the ^{1}H NMR spectrum of the fraction with published spectral data from authentic 2-(1*E*-pentadecenyl)furan (Fraga and Terrero, 1996). Corroboration of the proposed structure, 2-(1*E*-pentadecenyl)furan **2** (Figure 1), was obtained by synthesis.

The first of the minor components (2.8%), which eluted on GC (DB5-MS column) between the two major components, gave a molecular ion at *m/z* 276 in EI-MS and had an EI-MS very similar to that of 2-(1*E*-pentadecenyl)furan. Thus, the structure of this compound was tentatively assigned as the isomeric 2-(1*Z*-pentadecenyl)furan **3** (Figure 1), which was confirmed by synthesis. The *Z* isomer had a diagnostic one-proton multiplet at δ 5.58 (dt, J = 11.9, 6.9 Hz) due to the distal olefinic proton. To our knowledge, this is the first report of this compound.

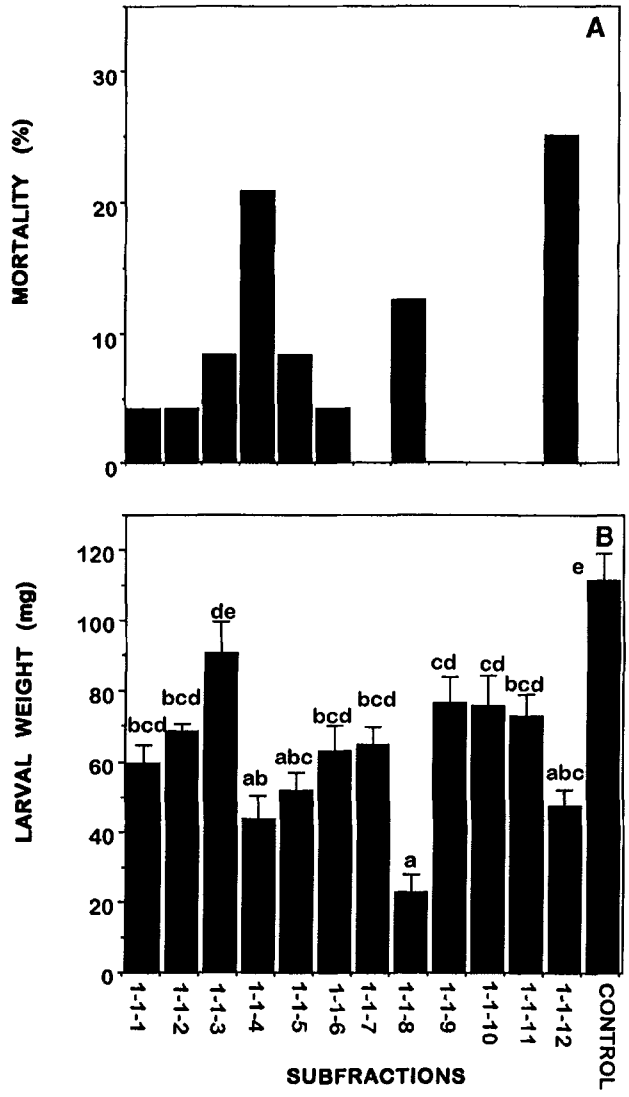


FIG 3. Percent mortality (A) and weight (B) of *S. exigua* larvae fed control diet and diet containing subfractions of fraction 1-1. Different letters indicate statistical differences between treatments (Tukey's pairwise comparisons, $P < 0.05$). Fractions were bioassayed at concentrations equivalent to 20 mg crude oil per milliliter artificial diet.

The second minor component (2.6%) gave an EI molecular ion at *m/z* 306, with a base peak at *m/z* 81, a large fragment at *m/z* 95, and a series of ions separated by 14 mass units (some weak ions in the series were missing), from *m/z* 95 to 263. On the basis of the spectral similarities to compound 1, this compound was assigned the previously unknown structure 2-(heptadecyl)furan **4** (Figure 1), which was confirmed by synthesis.

The final component in the fraction (6%) gave a molecular ion at *m/z* 304, a base peak at *m/z* 94, and a large fragment at *m/z* 107, indicative of a furan conjugated to a 1,2-disubstituted olefin. The mass spectrum was otherwise very similar to that of 2-(1*E*-pentadecenyl)furan. Consequently, the compound was tentatively assigned the structure 2-(1*E*-heptadecenyl)furan **5** (Figure 1).

The second active fraction, 1-1-8, was composed of a major and a minor component (ratio ~5:1). The major component gave a significant molecular ion at *m/z* 302 (14%), a base peak at *m/z* 81, and significant fragments at *m/z* 94 and 95 (36 and 34%) in EI-MS, again indicative of a substituted furan with an alkyl chain at the two position. The high-resolution exact mass measurement provided a molecular formula of $C_{21}H_{34}O$, corresponding to five sites of unsaturation, three of which were accounted for by the furan ring. The ^1H NMR spectrum contained a multiplet corresponding to four very similar olefin protons (δ 5.37), and a broad two-proton triplet at δ 2.78, assignable to a bisallylic methylene group. These data suggested that the two double bonds in the side chain might be in a (*Z*,*Z*)-1,4-diene structural unit typical of fatty acids. There were no multiplets indicative of branch points, allowing a tentative assignment of the structure as the known compound 2-(8*Z*,11*Z*-heptadecadienyl)furan **6** (Figure 1) (Murakoshi et al., 1976), and this structure was confirmed by synthesis.

The minor component of this fraction gave a molecular ion at *m/z* 300, with a molecular formula of $C_{21}H_{32}O$, suggesting that it was an analog of the major component with one further unsaturation. The base peak at *m/z* 94 and a large fragment at *m/z* 107 (73%) indicated that the additional double bond was conjugated with the furan ring, and this was supported by multiplets in the ^1H NMR of the mixed fraction at δ 6.12, 6.18, and 6.34, typical of a 1,2-disubstituted double bond of *E* configuration conjugated to the furan ring. No other peaks were seen in the olefin region of the ^1H NMR spectrum, other than those attributable to the major component, indicating that the signals due to the protons on the two remaining double bonds of the minor compound were hidden under the multiplet at δ 5.37. There data suggested that this compound had a (*Z*,*Z*)-1,4-diene structure also. On the sum of the evidence, the structure of the minor component of this fraction is tentatively proposed as 2-(1*E*,8*Z*,11*Z*-heptadecatrienyl)furan **7** (Figure 1).

The third fraction, 1-1-12, did not elute from the GC under standard conditions (max. oven temperature 300°C). On reverse-phase TLC plates, the com-

pound did not migrate with MeOH solvent, but did move up the plate with mixed MeOH–methylene chloride solvent mixtures, indicating a highly lipophilic compound. The compound was readily base hydrolyzed to produce primarily oleic acid, with smaller amounts of palmitic and palmitoleic acids. Similarly, reduction with $LiAlH_4$ produced oleyl alcohol, with lesser amounts of palmityl and palmitoleyl alcohols. These data were suggestive of a glyceride structure. The highest mass ion cluster seen in the FAB mass spectrum was centered at *m/z* 603, in the molecular weight range suggestive of a diglyceride. However, on silica gel TLC plates developed with hexane–ethyl acetate (10:1), 1,2-diolein and 1,3-diolein standards had R_f values of 0.10 and 0.14, respectively, versus the 0.51 R_f value of the unknown that corresponded with a triolein standard. Comparison of the ^1H NMR spectrum with an authentic standard confirmed that the major component of the fraction was indeed triolein **8** (Figure 1). In particular, there were two multiplets at δ 4.15 and 4.30, assignable to two pairs of diastereotopic protons on carbons 1 and 3 of the glyceride, with large geminal couplings to each other (J = 11.9 Hz) and smaller, unequal couplings (6 and 4 Hz, respectively) to the single proton (δ 5.27) on carbon 2 of the glyceride. The six olefinic protons appeared as an undifferentiated multiplet at δ 5.33.

The minor components of this fraction were not conclusively identified, but the evidence suggests that they are mixed triglycerides, with either palmitic or palmitoleic acids substituting for one or more of the oleic acid moieties of triolein.

Synthesis of Furan Compounds

Syntheses of the saturated C_{15} and C_{17} 2-(alkyl)furans **1** and **4** were achieved by the coupling of the appropriate bromoalkanes with 2-lithiofuran in THF. Both of the low-melting saturated alkylfurans could be recrystallized in methanol, allowing easy purification on multigram scale.

2-(1*E*-pentadecenyl)furan **2** and its *Z* isomer **3** were prepared by Wittig reaction of the ylide prepared from tetradecyl triphenylphosphonium bromide with furfural (Scheme 2B), to afford a 7:3 mixture of *Z* and *E* isomers (Lie Ken Jie and Lam, 1978). Photochemical isomerization of the mixed isomers in benzene with iodine catalysis (Ikedawa et al., 1970), followed by recrystallization from methanol, gave the *E* isomer **2** in greater than 97% purity.

In the first step of the synthesis of doubly unsaturated furan **6** (Scheme 3), linoleic acid **12** was treated with bromine to afford the tetrabromo acid **13**. Compound **13** was decarboxylated by a modification of the Hunsdiecker reaction to give pentabromide **14**. Regeneration of the diene moiety with zinc powder in THF yielded bromo diene **15**, which was converted to the **16**, and then

coupled with 2-lithiofuran to afford the doubly unsaturated avocadofuran **6** (Murakoshi et al., 1976).

Toxicity Studies

Table 1 and Figure 4 show the growth inhibitory and mortality effects of the most insecticidal compounds found in each of the three active subfractions (1-1-4, 1-1-8, and 1-1-12; Figure 3). 2-(Pentadecyl)furan **1**, present in subfraction 1-1-4, was the most active of the two major compounds from that subfraction. Furan **1** significantly inhibited larval growth, and reduced larval development by >70% at concentrations above 600 μg/g (Table 1). The LC_{50} [95% Fiducial limits (FL)] was 1031 μg/g (988–1084 μg/g) of furan **1** in diet, with a log dose-probit regression line slope of 7.44 ± 0.84 (Figure 4).

The other major component present in subfraction 1-1-4, 2-(1*E*-penta-

TABLE 1. GROWTH INHIBITORY AND MORTALITY EFFECTS OF SYNTHETIC COMPOUNDS FROM AVOCADO IDIOBLAST OIL CELLS TO *S. exigua* LARVAE

Concentration (μg/g)	7-Day larval weight (mg, mean ± SE)	7-Day instar (mean ± SE)[a]	GI[b]	EC (%)[b]	Mortality (%)
2-(Pentadecyl)furan					
600	8.50 ± 0.95b	2.29 ± 0.07c	0.69	72.89	9.37
750	4.44 ± 0.45ab	2.04 ± 0.07bc	0.55	85.83	18.75
900	3.03 ± 0.35ab	1.74 ± 0.07ab	0.26	90.35	32.29
1,050	1.41 ± 0.15a	1.61 ± 0.07a	0.25	95.50	54.17
1,200	1.32 ± 0.19a	1.56 ± 0.09a	0.15	95.78	71.87
2-(Heptadecyl)furan					
600	6.44 ± 0.44b	2.25 ± 0.06c	0.73	79.46	2.08
750	4.03 ± 0.44ab	1.95 ± 0.06b	0.62	87.14	4.20
900	3.19 ± 0.30a	1.87 ± 0.06b	0.56	89.84	10.42
1,050	2.02 ± 0.16a	1.65 ± 0.07ab	0.46	93.55	16.67
1,200	1.28 ± 0.16a	1.43 ± 0.08a	0.23	95.93	54.17
Triolein					
7,000	10.84 ± 1.42ab	2.35 ± 0.08c	0.64	65.43	18.75
8,000	17.67 ± 2.05b	2.70 ± 0.10bc	0.67	43.64	26.04
9,000	4.88 ± 0.65a	1.91 ± 0.08ab	0.39	84.44	40.63
10,000	5.79 ± 0.96a	1.87 ± 0.10a	0.37	81.52	42.71
11,000	8.82 ± 2.22ab	1.95 ± 0.13a	0.28	71.85	59.37
Control	31.35 ± 2.13c	3.19 ± 0.07d	1.03		3.57

[a]Treatments followed by the same letter within each compound are not significantly different from each other (Tukey's pairwise comparisons, $P < 0.05$).
[b]GI = growth index, EC = effective concentration; see text for calculations.

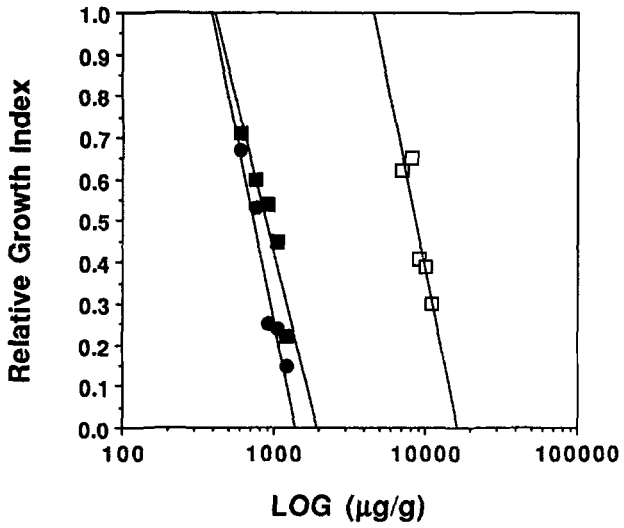


FIG. 4. Effects of synthetic avocadofurans or triolein on *S. exigua* larval development. Solid circles: response to 2-(pentadecyl)furan ($y = 5.66 - 1.79 * \log(x)$, $r^2 = 0.94$); solid squares: response to 2-(heptadecyl)furan ($y = 4.82 - 1.47 * \log(x)$, $r^2 = 0.89$); open squares: response to triolein ($y = 7.68 - 1.83 * \log(x)$, $r^2 = 0.86$).

decyl)furan **2**, showed biological activity only at concentrations approximately three times higher than furan **1**, inhibiting *S. exigua* growth at concentrations of 3500 µg/g or higher. At 3500 µg/g, larval growth was reduced by 57.3% compared to the controls, but mortality was only 12.5%.

2-(Heptadecyl)furan **4** was present as a minor component of subfraction 1-1-4, and it significantly inhibited larval growth (Table 1). Although mortality was low (<15%) at concentrations between 600 and 900 µg/g, larval development and growth were significantly reduced (>75%) at these concentrations (Table 1). The LC_{50} (95% FL) of 2-(heptadecyl)furan in diet was 1206 µg/g (1165–1273 µg/g), with a log dose-probit regression line slope of 14.03 ± 2.29 (Figure 4).

The major component in subfraction 1-1-8, 2-(8Z,11Z-heptadecadienyl)furan **6**, was active only at concentrations (1600 µg/g or higher) twice as high as the saturated analog **4**. At 1600 µg/g, *S. exigua* growth was inhibited by 54.51% compared to control larvae, but mortality was <5%.

Triolein, the major component in subfraction 1-1-12, significantly inhibited larval growth at relatively high concentrations (Table 1), but larval weight was not linearly correlated with concentration. Concentrations of 700 µg/g or higher significantly reduced larval weight and development (Table 1). The LC_{50} (95%

FL) of triolein in diet was 10,364 μg/g (9813–11,277 μg/g), with a log dose-probit regression line slope of 5.75 ± 0.94 (Figure 4).

DISCUSSION

Bioassay-driven fractionation of crude avocado idioblast oil resulted in the identification of five compounds that were toxic to larvae of *S. exigua*. Four of these belonged to the previously identified class of compounds known as avocadofurans, which, with one exception (the aquatic plant *Elodea canadensis* Michaux) (Previtera et al., 1985), have been found only in plants in the genus *Persea*. The fifth compound, the common triglyceride triolein, is a general constituent of avocado mesocarp and composes 15.8% of the total triglyceride composition of avocado mesocarp 12 days after flowering (Gaydou et al., 1987). Three other previously unknown avocadofurans (**3**, **5**, and **7**) were identified as minor components of fractions 1-1-4 and 1-1-8.

Kashman et al. (1969a, b) first reported the avocadofurans as a new class of phytochemicals. They isolated 2-(trideca-12-ynyl)furan and 2-(trideca-12-enyl)furan from *P. americana* fruit and seeds. Magalhaes et al. (1970) subsequently identified several other 2-alkylfurans with C_{13} mono- and diunsaturated side chains from methanol extracts of avocado seeds [*Persea gratissima* Gärtn. (syn. *P. americana*)] from Brazil. Several other avocadofurans have been identified since then (e.g., Néeman et al., 1970; Murakoshi et al., 1976; Weyerstahl et al., 1993; Fraga and Terrero, 1996).

The avocadofurans have received limited screening for biological activity to date. Néeman et al. (1970) tested a group of eight new long-chain aliphatic compounds from avocados, and some derivatives, for activity against 13 species of bacteria and a yeast and reported that 2-(trideca-12-enyl)furan inhibited growth of *Bacillus subtilis* (Ehrenberg) Cohn and *Staphylococcus aureus* Rosenbach. Murakoshi et al. (1976) tested 2-(8*Z*,11*Z*-heptadecadienyl)furan **6** produced by acid-catalyzed dehydration of persin from avocado leaves against silkworm larvae, *B. mori*, and found no activity at concentrations in the diet up to 300 μg/g. We found that *S. exigua* larvae were only moderately susceptible; a decrease in survivorship was found only at concentrations of **6** in excess of 1600 μg/g.

Although the avocadofurans we tested are structurally similar, our studies showed differences in their toxicity and growth inhibition effects. The two saturated avocadofurans **1** and **4** were more toxic and growth inhibitory to *S. exigua* than either of the unsaturated compounds **2** and **6**. Of these, **1** was the most toxic. Furthermore, these avocadofurans appear to be active as antifeedants at substantially lower (sublethal) concentrations, as demonstrated by the growth inhibition effects.

To our knowledge, triolein or other triglycerides have never been reported to have deleterious effects on insects, and the toxicity exhibited by triolein at levels of about 1% in artificial diet was unexpected. Furthermore, studies in progress indicate strong synergism between triolein and the avocadofurans (C. Rodriguez-Saona and J. Tromble, unpublished). The results of these studies will be published in due course.

At this early stage, the potential for avocadofurans as lead compounds for a new class of insecticides is unknown. There is no information available as to their nontarget (especially mammalian) toxicity, chemical stability, photostability, and phytotoxicity. However, the fact that the structures are simple and can be made in a single step from readily available precursors may provide impetus for further investigations.

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IDENTIFICATION AND DISTRIBUTION OF OVIPOSITION STIMULANTS FOR MONARCH BUTTERFLIES IN HOSTS AND NONHOSTS

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Abstract—Flavonol glycosides that act as oviposition stimulants for monarch butterflies were surveyed from a range of asclepiad hosts and some nonhosts. Major stimulants also were identified as quercetin-3-*O*-(2″-*O*-β-xylosyl)-β-D-galactoside and quercetin-3-*O*-β-D-galactoside from *Asclepias syriaca* and *A. incarnata*, respectively. The flavonol glycosides in *A. curassavica*, *A. tuberosa*, *A. incarnata*, *A. syriaca*, *A. humistrata*, *A. albicans*, *A. eriocarpa*, *Calotropis procera*, *Cynanchum acutum*, *Vincetoxicum* (*Cynanchum*) *nigrum* and in nonhosts *Hoya australis* and *Nerium oleander* were compared and characterized by HPLC and spectral studies. There was great variation in quercetin glycoside content. On the basis of the sugar moieties attached to quercetin, the asclepiad glycosides were classified broadly as those containing: (1) galactose, glucose, and rhamnose; (2) galactose, glucose, and xylose; and (3) galactose, glucose, xylose, and rhamnose. In most cases, galactose was attached to the 3-*O*-position (1″) of quercetin and other sugars were attached either to the 2″ or 6″ position of galactose. The sugars of triglycosides were attached at both 2″ and 6″ positions. A geographical pattern of flavonol distribution that may have affected the evolution of host recognition by the butterflies is suggested.

Key Words—*Danaus plexippus*, monarch butterflies, Nymphalidae, Asclepiadaceae, Apocynaceae, oviposition stimulants, flavonol glycosides, *Asclepias*, *Calotropis*, *Nerium*, *Cynanchum*, *Hoya*.

INTRODUCTION

The monarch butterfly, *Danaus plexippus* L., oviposits mainly on plants belonging to the family Asclepiadaceae, particularly within the genus *Asclepias* in

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North America (Ackery and Vane-Wright, 1984). The chemistry of Asclepiadaceae is diverse, and various classes of secondary metabolites have been reported. The cardenolides and, more recently, flavonoids have been shown to play a role in interactions with monarch butterflies. Cardenolides are sequestered by the monarchs and have been extensively studied (Zalucki et al., 1990). Recently, we reported quercetin glycosides from *Asclepias curassavica* that serve as oviposition stimulants (Haribal and Renwick 1996).

Studies of postalightment oviposition behavior of monarchs on three host species, *A. curassavica*, *A. incarnata*, and *A. tuberosa*, which vary widely in their cardenolide and flavonoid contents, showed that forelegs, mid-legs, and antennae are used for recognition and assessment of host suitability. Use of antennae often is preceded by the use of mid-legs, the tarsal claws of which rupture the leaf surface to release chemicals within the leaf. Perception of these occurs through antennal tapping. The use of forelegs alone can lead to substantial egg laying on *A. incarnata*. On *A. tuberosa* all appendages are used, and few eggs are laid on this least preferred host (Haribal and Renwick, unpublished). Electrophysiological studies indicate that receptors on different appendages have different sensitivities to behaviorally active flavonol glycosides (Baur et al., 1997).

The differential postalightment oviposition behavior of monarchs on different hosts prompted us to compare the flavonoid chemistry of some of these host plants. We identified active flavonoids from *A. syriaca* and *A. incarnata*, naturally occurring species in North America. We also compared HPLC profiles of flavonoid glycosides in other hosts and in nonhost milkweeds that are known to be hosts for other Danainae species.

METHODS AND MATERIALS

Insects

Insects were reared on *A. curassavica* in a greenhouse as previously described (Haribal and Renwick, 1996).

Plants

Most plants were grown from seeds in a greenhouse at 23 $\pm$ 2°C with supplemental lighting to provide a 16L:8D photoperiod. *A. incarnata* and *A. tuberosa* seeds were obtained from Thompson and Morgan. Seeds of *A. syriaca* and *Vincetoxicum* (*Cynanchum*) *nigrum* were collected around Ithaca, New York. Seeds of *A. albicans*, *A. eriocarpa* (Montgomery County, California), *A. speciosus* (Reno, Nevada), and *Cynanchum acutum* (Egypt) were provided by Dr. Brian Farrell. *A. humistrata* was collected from the Lake Delancy area in Ocala National Forest, Florida. *Calotropis procera* was collected from Ponce, Puerto

Rico. Foliage samples of *Hoya australis* and *Nerium oleander* were obtained from the Plant Sciences Department greenhouses at Cornell University, Ithaca.

Analysis of Plants for Flavonoids

A 5-g sample of plant tissue, generally young leaves, was extracted in boiling 95% ethanol, homogenized in a blender, and filtered. The solution was concentrated to 1 g leaf equivalent (gle)/ml, and diluted with an equal amount of water to precipitate chlorophyll and other insoluble materials. Samples were centrifuged and the clear supernatant solution filtered through 4-μm filters. A 0.1-gle amount (diluted to 2 ml with water) was injected directly into the HPLC column (C18 Bondex 10 μ, 30 $\times$ 0.78 cm), and flavonoids were separated with a water–acetonitrile gradient (Program 1-flow rate 3 ml/min: 0% acetonitrile at 10 min; 10% acetonitrile at 20 min; 15% at 30 min; 30% at 40 min; 100% at 50 min). Retention times were compared with those of authentic compounds. Flavonols were classified as quercetin or kaempferol derivatives on the basis of their UV maxima on a diode array detector system. A few samples were run in a slightly different program (program 2: water–acetonitrile gradient, flow rate 1 ml/min: 0% acetonitrile at 5 min; 20% at 10 min; 25% at 20 min; 30% at 40 min; 60% at 40 min; 100% at 50 min) to maximize separation of peaks. Duplicate samples of a few extracts of both young and old leaves of *A. incarnata*, *A. curassavica*, *A. syriaca*, and *A. tuberosa* were run to check for variation. We compared extracts for the presence of compounds behaviorally active as oviposition stimulants: **1a**, quercetin-3-*O*-(2″,6″-α-D-dirhamnopyranosyl)-β-D-galactoside, and **1b**, quercetin-3-*O*-(2″,6″-α-D-dirhamnopyranosyl)-β-D-glucoside; **2**, quercetin-3-*O*-(6″-β-D-glucosyl)-β-D-galactoside; **4**, quercetin-3-*O*-(6″-α-D-rhamnosyl)-β-D-glucoside (rutin) (Haribal and Renwick, 1996); and **3**, quercetin-3-*O*-(2″-α-D-rhamnopyranosyl)-β-D-galactoside, which has not yet been tested for activity. Compounds **5** and **6** were identified.

Isolation of Active Compounds from A. syriaca *and* A. incarnata

Extraction. Fresh young leaves of *A. syriaca* or *A. incarnata* were extracted in 95% boiling ethanol for 5 min. The material was blended in a heavy-duty Waring blender and the insoluble material removed by filtration. The resulting extracts were concentrated under reduced pressure almost to dryness, diluted with H_2O, and sequentially extracted with hexane, ethyl acetate, and *n*-butanol. The butanol extract showed the presence of flavonoids and was further chromatographed by HPLC on a C18 semi-prep column (Bondex, 10 μm 300 $\times$ 7.8 mm, Phenomenex). Fractions containing flavonoids were collected and individual compounds were purified by repeated HPLC. The butanol fraction of *A. syriaca* showed only one major flavonoid peak (**5**) (diode array detector, DAD). This was purified and identified. *A. incarnata* contained two quercetin flavo-

noids, compounds **5** and **6**. To obtain larger quantities of pure compounds for spectral and chemical analysis, the butanol fraction was fractionated on an open C18 rp flash column by using a water–methanol gradient. Compound **5** was further purified from the appropriate fraction by HPLC, and **6** was isolated after collection by repeated reprecipitation from methanol solution.

Acid Hydrolysis. Two to 3 mg of flavonoid glycoside was hydrolyzed with 2 N HCl for 4 hr at 80°C. The reaction mixture was extracted with ethyl acetate. The ethyl acetate fraction gave an aglycone, identified as quercetin by co-TLC. The aqueous fraction was evaporated to dryness and the products converted to TMS derivatives. The products were chromatographed on GC and retention times compared with those of TMS derivatives of standard sugars. The aqueous fraction also was cochromatographed with standard sugars on paper with C_6H_6–C_5H_5N–HOAc–H_2O (5 : 1 : 3 : 3) and *n*-butanol–HOAc–H_2O (4 : 1 : 5) solvent systems. Aniline hydrogen phthalate was used to visualize the sugars (Harborne, 1973).

GC-MS data were obtained from a capillary column (DB-5, 30 m × 0.23 mm) in a HP5890 GC linked to a HP 5970 MSD. All ^1H NMR were recorded at 399.99 MHz and ^{13}C NMR at 25.59 MHz in CD_3OD.

Bioassays. Artificial leaves were made from light green sponges cut with a bandsaw into blocks of approximately 45 × 85 × 5–6 mm and thoroughly washed with water to remove all chemicals. Test extracts, generally 0.5 gle, were dissolved in 3–3.5 ml water to provide the required concentrations and applied uniformly with a pipette. Control sponges were treated with an equal amount of solvent (water). Sponges were inserted vertically into 150-ml Erlenmeyer flasks filled with distilled water such that they were 3–4 cm into the water, to maintain the necessary moisture for oviposition. One control and one experimental flask were placed on an inverted plastic basket in a 1-m^3 cage provided with sucrose feeders, and an intact *A. curassavica* plant was placed under the basket as a source of host volatiles. Each experimental cage contained at least four females and four males that were 6–8 days old. Most of the females were previously mated. Males were included to ensure flight activity, as they often chased females from the corners of the cages. At the end of the test period (usually 6–10 hr), the eggs on both treated and control sponges were counted. Stimulatory activity was indicated when significantly more eggs were laid on treated sponges, based on statistical analysis with a one-tailed Wilcoxon's paired rank (non-parametric) test (Siegel and Castellan, 1988).

RESULTS

Flavonol Glycoside Content of Plants

Quercetin glycoside content varied from zero to 100% of the total flavonol glycosides. *A. syriaca* contained only one major flavonol (**5**), which accounted

for more than 95% of the total water soluble flavonols (Figure 1). *A. albicans* contained as many as 12 flavonol glycosides, quercetin, and kaempferol derivatives. Although few quercetin derivatives were present in *A. albicans*, the total concentration was nonetheless slightly higher than that of kaempferol derivatives. Five of the 10 species of *Asclepias* studied, as well as *Calotropis procera*, contained compound **5**. In some samples of *A. tuberosa*, a small amount of compound **5** also was detected. Triglycosides of quercetin (**1a** and **1b**, which could not be separated by HPLC) were present in *A. curassavica*, *A. eriocarpa*, and both species of *Cynanchum*. Rutin (**4**) was present only in *A. curassavica*, and at low concentration in *Nerium oleander*. In *Cynanchum acutum*, *V. Nigrum*, and *Calotropis procera*, only quercetin derivatives were detected. The representatives of the genera *Cynanchum* and *Vincetoxicum* also contained anthocyanins. The nonhost, *Hoya australis* and *Hibiscus* sp., did not contain any detectable quercetin derivatives.

On the basis of UV calibration curves, concentrations of **5** and **6**, in 0.5 gle of *A. incarnata* extract, were calculated to be about 44 μg (0.74×10^{-7} mol) and 275 μg (6.1×10^{-7} mol), respectively. Similarly, the concentration of compound **5**, in 0.5 gle extract of *A. syriaca*, was approximately 250 μg (4.2×10^{-7} mol).

In general, the content of quercetin glycosides in preferred hosts was higher than that of kaempferol glycosides. In samples of *A. tuberosa*, flavonols were minimal or undetectable. Plants that received eggs contained at least a small quantity of flavonoids. We also found that younger leaves of hosts had two to four times more total flavonoids than older leaves. Levels of quercetin derivatives and total kaempferol derivatives are summarized in Table 1.

Bioassays of Compounds 5 and 6

Compounds **5** and **6** were tested in choice assays. A mean of 175.0 ± 50.0 (SE) eggs were deposited on sponges treated with compound **5** while controls received a mean of 72.5 ± 17.5 (SE) eggs (Table 2). Similarly, the sponges treated with compound **6** received a mean of 142.4 ± 54.2 (SE) eggs and controls 54.8 ± 10.0 (SE). The fact that treatments received more eggs than respective controls indicated that the compounds are active as oviposition stimulants (Table 2).

Identification of Active Compounds 5 and 6 from A. incarnata *and* A. syriaca

Compound 5. The UV spectrum of **5** in MeOH gave λ_{max} at 255, 265sh, 296sh, and 356 nm, typical of a quercetin derivative. UV shift reagent studies with $AlCl_3$ (λ_{max} at 452, 355, 292 nm); $AlCl_3$ + HCl (424, 376, 290 nm); NaOAc (λ_{max} at 377, 342sh, 260.7 nm); and NaOAc + H_3BO_3 (376.5, 322, 271 nm) indicated that **5** was a 3-*O*-substituted quercetin glycoside. The FAB mass spectrum gave M + 1 at 597 for a possible molecular formula of $C_{26}H_{28}O_{16}$,

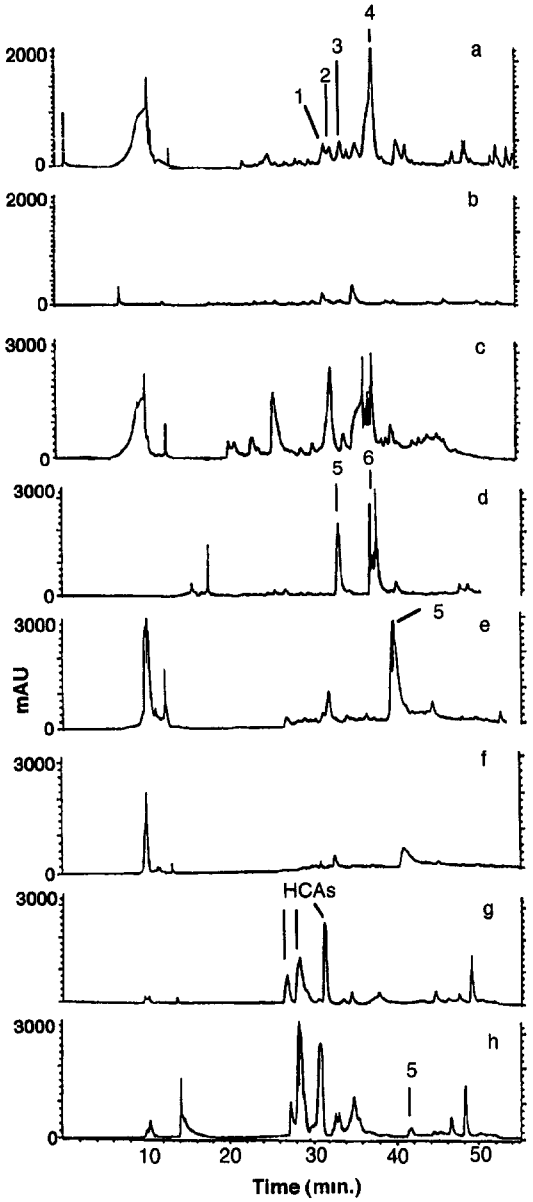


FIG. 1. HPLC chromatograms of *Asclepias* species: (a) *A. curassavica* young leaves, (b) *A. curassavica* old leaves, (c) *A. incarnata* young leaves, (d) *A. incarnata* old leaves, (e) *A. syriaca* young leaves, (f) A. Syriaca old leaves. (g) *A. tuberosa* young leaves, and (h) *A. tuberosa* old leaves. Program 1 was used to analyze samples a–d and program 2 for e–h. HCAs: hydroxycinnamic acid derivatives. Numbers correspond to compounds identified in Figure 2.

TABLE 1. QUANTITATIVE COMPARISON OF WATER-SOLUBLE QUERCETIN AND KAEMPFEROL GLYCOSIDES IN HOSTS AND NONHOSTS OF MONARCH BUTTERFLY

Plant species	Relative concentrations of quercetin glycosides (based on integration of HPLC peaks)							Total quercetin glycosides	Total kaempferol glycosides	Remarks[a]
	1 (1a and 1b)	**2**	**3**	**4**	**5**	**6**	Others			
Asclepias syriaca					194.3			194.3	48.9	E and F
A. syriaca (O)[b]					53.0			53.0	1.8	E and F
A. incarnata					48.9	165.3		214.2	1.1	E and F
A. incarnata (O)					66.9	108.2		175.1	10.9	E and F
A. albicans		31.1			25.2		22.4	78.7	73.0	E and F
A. humistrata		78.2			8.6			86.8	14.8	E and F
Calotropis procera			11.4		223.2			234.6	0.0	E and F
A. speciosus		11.6	153.7				39.0	204.3	0.0	E and F
A. eriocarpa	129.5		96.4					225.9	14.8	E and F
Cynanchum acutum	13.2		96.2					109.4	14.9	E and F[c]
Vincetoxicum nigrum	18.2							18.2	22.3	E and NF[d]
A. curassavica	16.6	14.4	17.9	74.6		19.4		126.3	80.1	E and F
A. curassavica (O)		8.8	3.4	19.1				31.3	26.2	E and F
A. curassavica flowers	14.4		25.3	34.6		22.2	24.8	96.5	20.6	E and F
A. tuberosa 1			2.2					2.2	0.0	E and F
A. tuberosa (O)						52.2	18.0	52.2	0.0	NE
A. tuberosa 2								0.0	0.0	NE
Nerium oleander						51.5		51.5	31.4	NE
Hoya australis								0.0	31.4	NE
Hibiscus sp.								0.0	33.1	NE

[a] E = eggs received by the plants; F = Larvae feed on the plants. NE = No eggs were received.
[b] O = Old leaves.
[c] Insects could be reared only to 3rd instars as there was not enough plant material to rear them to pupation.
[d] Eggs were laid on the plant but larvae did not feed on the plant.

TABLE 2. OVIPOSITION RESPONSES OF MONARCH BUTTERFLIES TO *A. incarnata* AND *A. syriaca* COMPOUNDS

	Eggs (mean ± SE/sponge)		Total females (*N*)	No. of Tests (*N*)	*P*[a]
	Treated	Control (water)			
Compound **5**	175.0 ± 50.0	72.5 ± 17.5	59	8	0.0534
Compound **6**	142.4 ± 54.2	54.8 ± 10.0	47	5	0.0313

[a]Wilcoxon's paired sign rank test was used to analyze the data on the basis of eggs laid on the treatment and control sponges in paired choice bioassays. Concentration of compound **5** was approximately 4.2×10^{-7} mol (250 μg) and concentration of compound **6** was approximately 6.1×10^{-6} mol (275 μg) per sponge.

which would suggest a diglycoside. Hydrolysis with 2 N HCl gave two sugars and quercetin. The sugars were identified as xylose and galactose on the basis of Co-PC with standards in two solvent systems and by comparison of retention times of their TMS derivatives on GC-MS with those of TMS derivatives of standard sugars.

The ^{1}H NMR gave peaks at δ 6.18, 6.35, 6.85, 7.72, and 7.78 ppm for protons attached to an aglycone moiety, consistent with a 3-*O*-substituted quercetin. Anomeric sugar protons appeared at δ 5.39 (d, J = 7.6 Hz) and 4.85 (d, J = 6.8 Hz) ppm for galactosyl and xylosyl moieties, respectively. The coupling constants for anomeric protons (7.6 and 6.8 Hz) indicated that both sugars were in the β-pyranosyl form. ^{13}C NMR gave peaks at δ 101.56 and 105.80 that were assigned to anomeric carbons of the galactose and xylose anomeric carbons, respectively (Table 3). Final complete assignments were made on the basis of 2D NMR (GHMQC and DQCOSY). Compound **5** was identified as quercetin 3-*O*-(2″-β-D-xylopyranosyl)-β-D-galactopyranoside, and this was confirmed by comparison of spectral data with reported literature values. (Larsen et al. 1982)

Compound 6. Compound **6** also was found to be a 3-*O*-substituted quercetin glycoside on the basis of its UV spectra. ^{1}H and ^{13}C NMR were indicative of a monoglycoside. Acid hydrolysis gave quercetin and a single sugar, which was identified as galactose by the same methods as for compound **5** (PC, GC-MS, and comparison with authentic samples) and by comparing ^{13}C NMR data (Table 3) with values reported in the literature (Webby and Markham, 1990).

DISCUSSION

The quercetin 3-*O*-(2″-β-D-xylopyranosyl)-β-D-galactopyranoside and quercetin 3-*O*-β-D-galactopyranoside identified as oviposition stimulants from

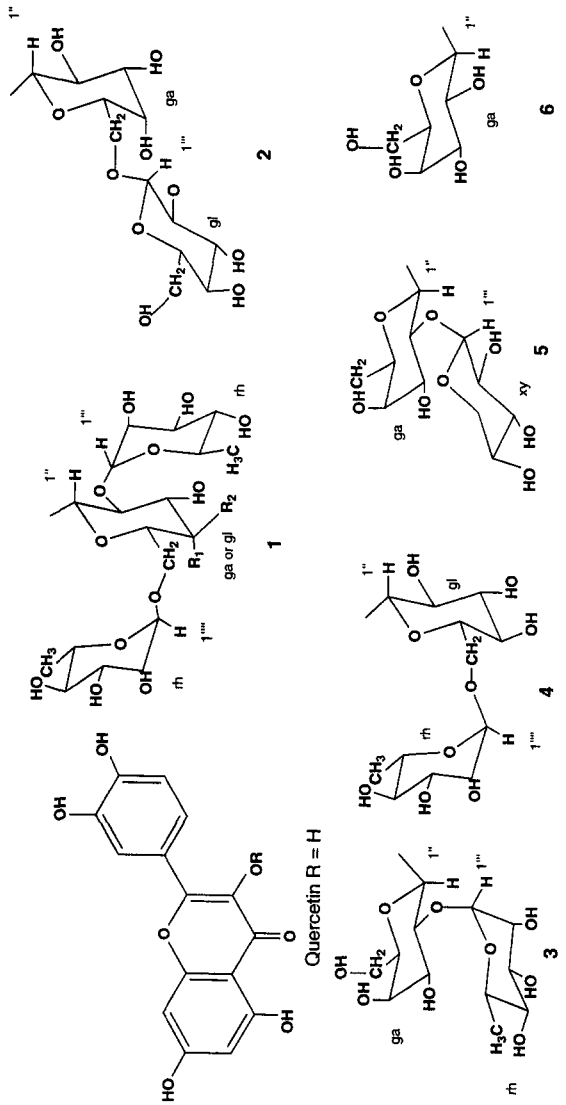
TABLE 3. NMR DATA OF COMPOUNDS **5** AND **6**

	5			6	
Carbon	^{13}C NMR	^{1}H NMR	*J* values	^{13}C NMR	^{1}H NMR
2	158.5			156.2	
3	135.3			133.4	
4	179.8			177.4	
5	160.0			161.2	
6	99.9	6.18	d, 2.4 Hz	98.6	6.20
7	166.0			164.0	
8	94.7	6.35	d, 2.4 Hz	93.4	6.41
9	158.3			156.2	
10	105.5			103.8	
1′	123.1			121.9	6.86
2′	116.2	6.85	bs	115.8	
3′	146.1			144.8	
4′	149.9			148.2	
5′	117.5	7.72	dd, 2.0 Hz, 8.4 Hz	115.8	7.59
6′	123.5	7.78	d, 8.4 Hz	121.0	7.84
1″	101.5	5.20	d, 7.6 Hz	101.7	5.17
2″	80.2	4.02	dd, 7.6, 9.2 Hz	71.1	3.85
3″	75.2	3.72	dd, 3.4 Hz, 9.8 Hz	73.1	3.82
4″	70.1	3.85	dd, 3.4 Hz	67.8	3.64
5″	77.1	3.43–3.45		75.8	3.56
6″	62.1	3.62	dd, 6.2 Hz, 11.2 Hz	60.1	3.47
1‴	105.8	4.80	6.8 Hz		
2‴	74.9	3.40	dd		
3‴	71.1	3.50	dt, 7.2 Hz, 2.8 Hz		
4‴	77.0	3.49	m		
5‴	66.5	3.25 and 3.95	dd, 9.6 Hz, 11.6 Hz, dd, 4.8, 11.6 Hz		

A. syriaca and *A. incarnata* differ from the active glycoside from *A. curassavica* (Haribal and Renwick, 1996). This means that monarch butterflies depend on a range of closely related compounds to recognize suitable hosts, although structural similarities between the active compounds are quite evident (Figure 2).

All active compounds are 3-*O*-substituted quercetin galactosides, and all have at least one substitution at position 2″ of the galactose attached to quercetin in the diglycosides. Except in **4** (rutin) and **2**, substitution is at the 6 position of the 1″ galactose (Figure 2). A requirement for activity, therefore, appears to be that the flavonoid must be a galactoside, preferably with substitution at the 2″-*O* position.

The identification of different compounds as stimulants from different hosts



1a. R1= OH, R2= H; R = 2'',6'', α-L-dirhamnopyranosyl β-D-galactopyranosyl
1b. R1= H, R2= OH; R = 2'',6'', α-L-dirhamnopyranosyl β-D-glucopyranosyl
2. R = β- D-glucopyranosyl (1 → 6) β-D-galactopyranosyl
3. R = α-L-rhamnopyranosyl (1→ 2) β-D-galactopyranosyl
4.R = α-L-rhamnopyranosyl (1 → 6) β-D-galactopyranosyl
5. R = Xylosyl (1 → 2) β-D-galactopyranosyl **6**. R = β-D-Galactosyl

FIG. 2. Chemical structures of flavonoids isolated from *Asclepias* spp.

might explain the observed variable butterfly behavioral patterns (Haribal and Renwick, 1997 unpublished). On *A. incarnata*, the use of forelegs alone can lead to substantial egg-laying, while on *A. curassavica*, the use of antennae seems to be important. Electrophysiological studies of behaviorally active flavonoids indicated that sensilla on the antennae were sensitive to **2** at low concentrations, while foreleg sensilla were stimulated by this compound only at higher concentrations. Sensilla of tarsomers 2, 3, and 4 of mid-tarsi were responsive to **1a** only at the 1 gle level, and sensilla of antennae did not respond. However, the total butanol fraction of *A. curassavica* containing mixtures of flavonol glycosides was a good stimulant at lower concentrations for sensilla on these appendages (Baur et al., 1998).

Most of the eggs were laid on young leaves near the top or on the flowers of *Asclepias* spp. (Haribal and Renwick, unpublished): One reason for this could be that younger tissues have higher concentrations of flavonoids than older lower leaves. In a three-choice test for oviposition preference among *A. incarnata*, *A. curassavica*, and *A. tuberosa*, *A. tuberosa* was least preferred. This can be explained by the fact that some samples of *A. tuberosa* lacked flavonoids, but instead were rich in hydroxycinnamic acid derivatives (HCAs).

The family Asclepiadaceae consists of more than 250 genera (Liede and Albers, 1994; Good, 1952), of which 45 are known to be hosts for Danainae butterflies (Ackery and Vane-Wright, 1984). Fifteen genera occur in the New World and some, such as *Asclepias*, *Cynanchum*, *Secamone*, and *Vincetoxicum* occur also in the Old World. The genus *Asclepias* is highly diversified in North America and consists of more than 100 species (Woodson, 1954). Some species, such as *A. curassavica* from the neotropics and *A. (Gomphrocarpus) fruticosa* from Africa, recently have become naturalized in many parts of the world. *Asclepias* has been extensively studied for its cardenolide content, but few reports are available on its flavonoids. Rahaman and Wilcock (1991) investigated flavonoids of about 20 species of asclepiads by TLC and reported that five of these produced both kaempferol and quercetin flavonoids while another eight species contained either quercetin or kaempferol. The presence of flavonoids in *C. procera* also was reported, but no details were provided (Mossa et al., 1991).

Wyatt and Hunt (1991), who studied four species of *Asclepias* (*A. exaltata*, *A. syriaca*, *A. purpurascens*, and *A. quadrifolia*) and their hybrids in North America, reported quercetin 3-*O*-(2″-xylosyl) glucoside as the major compound along with minor amounts of a triglycoside and quercetin 3-*O*-glucoside in *A. syriaca*. However, these results were based on cochromatography and hydrolysis and did not include any spectroscopic data to verify postulated structures. We did not detect quercetin 3-*O*-(2″-xylosyl)-glucoside in our own *A. syriaca* samples. Wyatt and Hunt (1991) also reported that *A. quadrifolia* was rich in quercetin 3-*O*-(2″-xylosyl)-glucoside, and *A. purpurascens* was shown to contain six flavonols, the most abundant of which were kaempferol glycosides.

On the basis of the quercetin flavonoid patterns obtained, the host plants of *D. plexippus* can be divided broadly into three categories (Figure 3), i.e., those with: (1) rhamnose, galactose, and glucose; (2) xylose, galactose, and glucose, and (3) rhamnose, xylose, galactose, and glucose. A pattern of sugar occurrence and distribution can be envisaged where the species from the northeastern United States and Canada (*A. syriaca*, *A. incarnata*, and *A. exaltata*) and southeastern United States and Canada (*A. humistrata*) contain flavonol glycosides with xylose, glucose, and galactose as major sugars, while plants from the western United States and Mexico (*A. eriocarpa*, *A. speciosa*) contain glycosides with rhamnose, glucose, and galactose. The southern plants, such as *A. curassavica* and *Calotropis procera*, have glucose, rhamnose, galactose, and xylose (Figure 3). This suggests that as *Asclepias* colonized the temperate zone

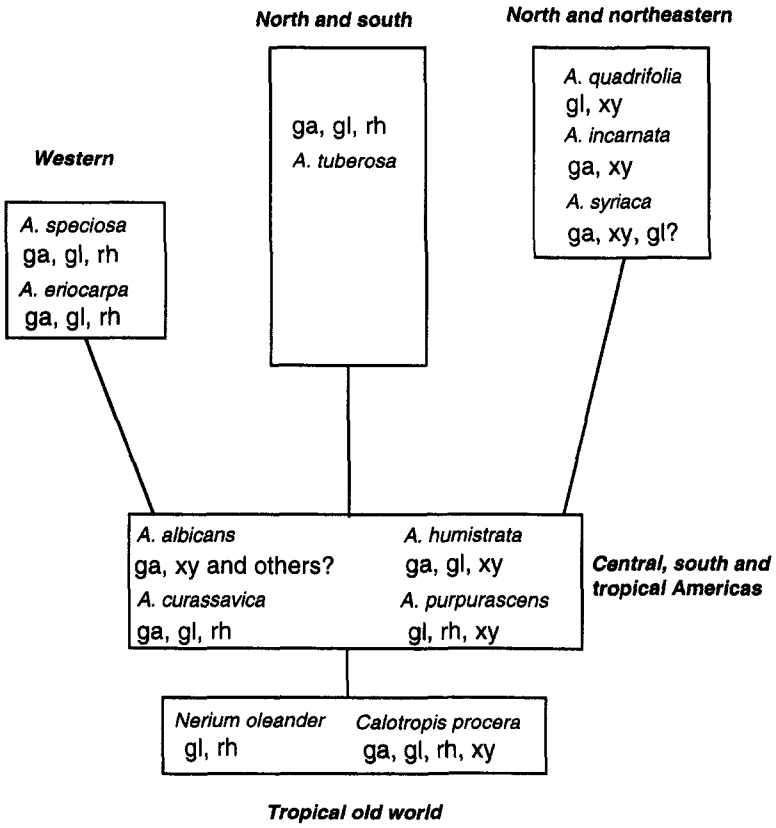


FIG. 3. Geographical distribution of asclepiadaceous plants and occurrence of flavonoid sugars. ga = galactose; gl = glucose; rh = rhamnose; xy = xylose.

of North America, the plants in the east evolved differently from the plants in the west. Woodson (1954) considers the series Incarnatae as a primitive group to which *A. curassavica* and *A. incarnata* belong. *Calotropis procera* is an Old World plant, indigenous to middle-eastern Asia, from where it has spread into the tropics of both the New and Old Worlds. All the species we investigated (and *Asclepias* reported in literature)—*A. incarnata*, *A. tuberosa*, *A. exaltata*, *A. quadrifolia*, *A. syriaca*, and *A. purpurascens*—belong to the Appalachian and Ozarkian regions. *A. humistrata* is centered in the Floridian region (Woodson, 1954). *A. speciosa* belongs to the Mexican region and *A. eriocarpa* and *A. albicans* are from the Californian region, the latter being from southern Baja California. Our observations of sugar patterns confirm the distinct geographical separation of these *Asclepias* species and point to distinct chemical differences.

The differences in flavonoid content of different asclepiad species may reflect their evolutionary history and their utilization by monarchs. Danainae butterflies occur primarily in tropical regions and are most diversified in the Old World (Ackery and Vane-Wright, 1984). The subgenus *Danaus* is mainly represented in tropical Central and temperate North America. *Danaus plexippus* is the only member of the genus found in both zones, as far as 50°N latitude. The spread of *D. plexippus* and *Asclepias* to the eastern and northeastern United States is a comparatively recent event, which occurred only after the deforestation of this region by European settlers (Brower, 1995). In the last 200 years, it has also become established in many other parts of the world, including the Pacific islands, southeastern Australia, and southwestern Europe. This distribution suggests that *D. plexippus* evolved in tropical regions, and as *Asclepias* spp. evolved to tolerate temperate climatic conditions and spread farther north, the butterfly coevolved with it. Recognition of host plants by specialist butterflies depends on the unique chemistry of these plants.

Other butterflies also have developed close relationships with their host plants based on specific chemistry. For example, different species of swallowtail butterflies that oviposit on the same *Citrus* hosts seem to have evolved to recognize a set of different compounds present as oviposition cues (Nishida, 1995). In fact, structural isomers and derivatives of stimulants have been shown to be deterrents, but in the case of monarchs, which feed on chemically different hosts and use three appendages to recognize their hosts, the variation in host chemistry may have led to evolution of differential chemical sensitivity on different appendages. This may also be correlated with the migratory behavior of the butterflies.

When *V. nigrum* was offered to monarchs in choice tests, some eggs were laid, but the first instar could not survive. This indicates that the plant contains oviposition stimulants but is not suitable for larval feeding and development. *V. nigrum* and *V. rossicum* have become major concerns to ecologists and natural area managers because of their aggressive spreading. These introduced weeds are replacing natural vegetation in many parts of the northeastern United States

(Sheeley and Raynal, 1996). In some locations, *V. nigrum* has replaced most of the natural vegetation, including *Asclepias*. spp. This may represent a threat to natural populations of monarch butterflies, which lay eggs on these plants but subsequently will perish in the absence of their *Asclepias* hosts.

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GEOGRAPHIC AND TEMPORAL VARIATION OF CARDENOLIDE-BASED CHEMICAL DEFENSES OF QUEEN BUTTERFLY (*Danaus gilippus*) IN NORTHERN FLORIDA

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Abstract—The cardenolide-based chemical defenses of danaine butterflies vary macrogeographically. This study demonstrates that these defenses also vary both microgeographically and temporally. We sampled 280 queen butterflies (*Danaus gilippus*) at 11 sites in northern Florida during the summer of 1993 and determined their cardenolide concentrations and thin-layer chromatography profiles. Queens captured in coastal salt marshes and xeric inland sites were low in cardenolide concentration, while those from hydric inland sites had much higher concentrations. Concentrations also varied temporally, especially at the coastal sites. Thin-layer chromatography analyses of wild-captured queens and those reared on local milkweeds indicated that microgeographic and temporal variation in the cardenolide chemistry of the butterflies is mediated by their host plants. The large differences in cardenolide concentrations among queen populations must result in strong differences in palatability spectra to vertebrate predators. This finding has major implications both for interspecific mimicry and for automimicry.

Key Words—Danainae, *Danaus gilippus*, queen butterfly, milkweed, Asclepiadaceae, cardenolide, cardiac glycoside, geographic variation, temporal variation, unpalatability, palatability spectrum, chemical defense, host plant, sequestration, thin-layer chromatography fingerprinting, mimicry, automimicry, Florida.

INTRODUCTION

Many insect species possess chemical defenses that make them unpalatable to predators (reviewed in Blum, 1981; Brower, 1984; Huheey, 1984; Whitman et

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al., 1990). Within single species, the amounts and types of chemical defenses can vary among individuals, resulting in a palatability spectrum (Brower et al., 1967, 1968). Palatability spectra are important, for they can influence the behavior of predators toward chemically defended prey (Fink and Brower, 1981), the survival of prey (Brower, 1984), and the effectiveness of mimicry and automimicry (Brower et al., 1970, 1971; Pough et al., 1973; Guilford, 1994).

Many herbivorous insects sequester chemical defenses from host plants (reviewed in Rothschild, 1972; and Bowers, 1990), which often vary in the amount and type of secondary chemicals that they produce (Chew and Rodman, 1979; McKey, 1979; Rhoades, 1979; Bernays and Chapman, 1994). Moreover, this variation is a primary cause of palatability spectra in herbivorous insects capable of sequestration (Roeske et al., 1976; Bowers, 1980; reviewed in Bowers, 1988).

Host-plant species frequently differ in both geographic distributions and secondary chemistries and thus can mediate geographic variation in the chemical defenses of herbivorous insects. While numerous studies have demonstrated chemical defense variation on a macrogeographic scale (Brower et al., 1973; Brower and Moffitt, 1974; Cohen, 1985; Ritland, 1991a; Bowers and Williams, 1995), few have looked for microgeographic differences (Eggenberger and Rowell-Rahier, 1991; Pasteels et al., 1995). These differences may occur commonly in nature and, in turn, may lead to variation in the dynamics of mimetic relationships at the local level, i.e., between sites separated only by a few kilometers, as hypothesized by Ritland (1991a, b, 1994). Insect chemical defenses also can vary over time (Malcolm and Brower, 1989; Eggenberger et al., 1992) due to seasonal shifts in available hosts (Malcolm and Brower, 1989).

This paper investigates geographic and temporal variation of the cardenolide contents of the queen butterfly [*Danaus gilippus* Cramer (Nymphalidae: Danainae)] in northern Florida. *Danaus gilippus* occurs from Argentina to the southern United States, and occurs from Florida westward to California (Opler and Krizek, 1984). In peninsular Florida, it is a multivoltine, year-round resident (Ritland, 1991a). Although some queens disperse northward during the summer (Scott, 1986), local breeding populations grow in size throughout the summer (Opler and Krizek, 1984; Ritland, 1991a). In Florida, queen larvae feed upon milkweeds (Family Apocynaceae, Subfamily Asclepiadoideae) (Judd et al., 1994), which often possess secondary chemicals called cardenolides that the queens can sequester and use as chemical defenses (Ritland, 1994). Florida is home to six genera and 36 species of milkweeds, which differ in their habitat requirements, season of availability (Long and Lakela, 1971; Wunderlin, 1982; Clewell, 1985), and presumably also in their cardenolide chemistries.

We present evidence that both small-scale geographic and seasonal differences in the milkweed flora are reflected in the amounts and kinds of cardenolides in the adult butterflies. This paper is thus the first to show that the

chemical defenses of a danaine vary between populations separated by only a few kilometers and also provides the first evidence that variation occurs at the same sites through a significant portion of the breeding season. We address the implications of this variation to the palatability of queens and to their mimetic relationships.

METHODS AND MATERIALS

Protocol for Sampling Wild Queens

From May to September of 1993, we captured 280 adult butterflies at 11 sites in three distinct ecozones (coastal, hydric inland, and xeric inland) in Levy and Dixie counties, Florida (Table 1, Figure 1). We achieved our goal of capturing approximately 10 queens at each site during each month, except in the xeric inland ecozone, where we did not find populations of five or more adults until late August (Table 2). Data from the nine queens captured on August 30 were grouped with the data for September.

Descriptions of Ecozones

Coastal Ecozone. As indicated in Figure 1, this ecozone is located along the southern portion of the Big Bend region of Florida, adjacent to the Gulf of Mexico. It is primarily salt marsh, but also includes patches of coastal hammock. Queens were sampled from sites 1–4, each of which was approximately 0.5 ha.

TABLE 1. LOCATIONS OF QUEEN SAMPLING SITES

Site	Latitude (N)	Longitude (W)	USGS quadrangle[a]
1	29°39′23″	83°24′15″	Steinhatchee
2	29°23′45″	83°12′09″	Shired Island
3	29°07′05″	82°46′07″	Withlacoochie Bay
4	29°06′47″	82°45′49″	Withlacoochie Bay
5	29°42′20″	83°17′25″	Jena
6	29°33′00″	83°10′55″	Cross City SW
7	29°15′55″	82°52′52″	Chiefland SW
8	29°10′45″	82°38′07″	Lebanon Station
9	29°27′00″	82°38′25″	Bronson
10	29°20′17″	82°30′42″	Bronson SE
11	29°25′59″	82°24′57″	Williston

[a]To find the site, refer to the 1:24,000 quadrangle topographic maps for the state of Florida (United States Geological Survey).

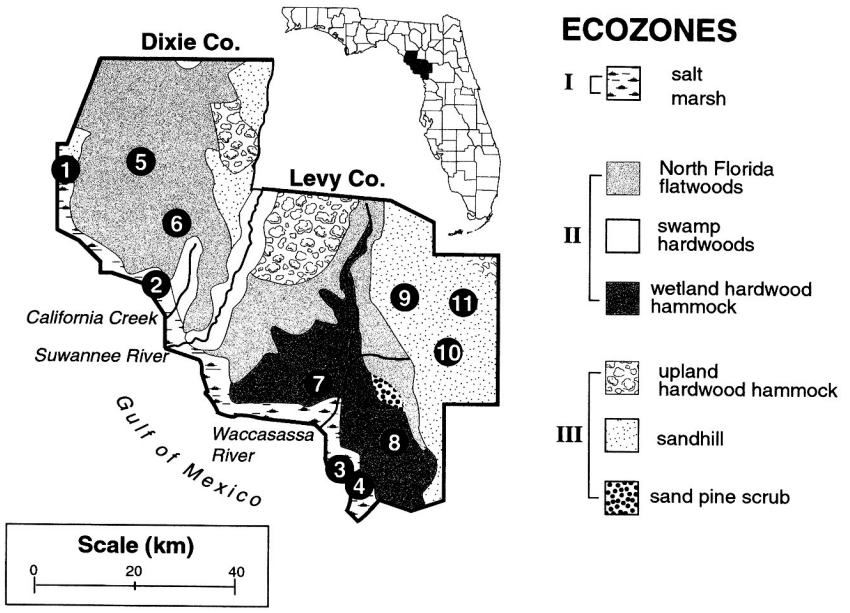

FIG. 1. Locations of three Florida ecozones and 11 study sites in Dixie and Levy counties. Boundaries of the seven plant communities in these ecozones follow the US Soil Conservation Service (1980). Ecozones are indicated by roman numerals: I, the coastal ecozone (sites 1–4); II, the hydric inland ecozone (sites 5–8); and III, the xeric inland ecozone, (sites 9–11).

The low elevation of these sites ensures occasional inundation by salt water, which plays a large role in structuring the plant communities of the salt marsh (Montague and Wiegert, 1990). The salt-marsh milkweed vine, *Cynanchum angustifolium* Pers., was abundant at all four coastal sites. Two other milkweed vines, leafless cynanchum (*C. scoparium* Nutt.) and angle-pod [*Matelea gonocarpa* (Walt.) Shinners], were found near site 3 on the islands of coastal hammock that dot the salt marsh.

Hydric Inland Ecozone. This zone is a wide band of lowlands between the salt marsh of the Gulf coast and the xeric uplands of the eastern portions of Dixie and Levy counties (Figure 1). The lowlands consist of a matrix of North Florida flatwoods, swamp hardwoods, and wetland hardwood hammocks, with occasional cypress ponds and marl prairies (Ewel, 1990; Florida Natural Areas Inventory, 1990). According to Fernald (1981), the hydric inland lowlands of Dixie and Levy counties possess flatwoods soils, which are poorly drained

TABLE 2. RECORD OF 280 WILD QUEENS SAMPLED AT 11 SITES IN LEVY AND DIXIE COUNTIES FROM JUNE THROUGH SEPTEMBER 1993

Sample dates	Captured (*N*)
Site 1	
26 June	10
24 July	10
19 Aug.	10
22 Sept.	6
Site 2	
6 June	9
8 July	10
14 Aug.	10
11 Sept.	11
Site 3	
14 June	12
6 July	11
5 Aug.	10
5 Sept.	8
Site 4	
19 June	9
18 July	10
21 Aug.	10
13 Sept.	10
Site 5	
24 July	10
19 Aug.	16
22 Sept.	2
Site 6	
14 Aug.	10
11 Sept.	10
Site 7	
20 July	15
18 Aug.	10
16 Sept.	14
Site 8	
13 Aug.	10
13 Sept.	2
Site 9	
3 Sept.	5
16 Sept.	2
Site 10	
15 Sept.	9
Site 11	
30 Aug.	9

spodosols that retain moisture. For detailed characterizations of these ecosystems, see Abrahamson and Hartnett (1990) and Ewel (1990).

Sites 5–8 were located at least 5 km from the Gulf coast and are unlikely to be inundated by salt water, except for the once-in-a-century storm surge caused by level 5 hurricanes coming from the Gulf (Fernald, 1981). Supporting this is the fact that the salt marsh milkweed, *C. angustifolium*, was not found at any of these sites. In contrast, *Asclepias perennis* Walt. and *A. lanceolata* Walt., two milkweeds known to prefer freshwater wetland habitats (Wunderlin, 1982; Clewell, 1985), were found at all four sites. A third milkweed, *A. longifolia*, was not found at any of these sampling sites, but was found only 30 m from site 5.

Xeric Inland Ecozone. In Dixie and Levy counties, this ecozone is composed of three plant communities: upland hardwood hammock, sandhill, and sand pine scrub (Figure 1). For detailed characterizations, see Myers (1990). Sites 9–11 are located more than 30 km from the Gulf of Mexico, on the northern portion of the Brooksville Ridge, where the dry, sandy soil is made up primarily of entisols (Fernald, 1981). The milkweed species found in this ecozone were *A. humistrata*, *A. tuberosa*, and *A. verticillata* L. For detailed descriptions of all eleven sites, see Moranz (1996).

Analysis of Cardenolide Contents of Wild Queen Butterflies

Captured butterflies were placed in individual envelopes and put into a portable ice chest, then frozen in a conventional laboratory freezer. Within 14 months of capture, the butterflies were dried at 60°C for 16 hr in a forced-draft drying oven. Anatomical measurements (dry weight and right forewing length) were made on the dried queens, as were determinations of wing condition and the presence or absence of beak marks on the wings. We scored wing condition on a scale from 1.0 (perfect) to 5.0 (worn, having lost many scales) in increments of 0.5. Right forewing length (to the nearest 0.5 mm) was measured with a ruler from the tip of the wing to the white spot at the base of the wing. Next, we ground and extracted each butterfly in petroleum ether (Walford, 1980; May, 1992) to determine the lipid content of each butterfly, and to remove the lipids from the samples. A portion of each sample was extracted in ethanol and spectroassayed to determine the cardenolide concentration in micrograms (equivalent to digitoxin) per 0.1 g of dry material; the spectroassay was performed in the manner of Malcolm et al. (1989), after Brower et al. (1972, 1975), with a Perkin Elmer Lambda IIs dual-beam spectrophotometer.

The remaining ethanol–cardenolide extract was cleaned for thin-layer chromatography (TLC) by the following procedure. Dried ethanol extracts were reliquefied with 2 ml distilled water and 1 ml ethanol and then partitioned three times with 2 ml 2:1 chloroform–ethanol. Contaminants were discarded in the

30% ethanol fraction, leaving cardenolides in the chloroform fraction. This fraction was dried under a nitrogen evaporator, reliquefied with chloroform, and filtered through a 0.5-μm Millipore filter on a Luer-lock syringe (Malcolm, 1990). The cleaned sample was analyzed by TLC with a 90:6:1 chloroform–methanol–formamide (CMF) solvent system, as in Brower et al. (1982) and Malcolm et al. (1989). Two cardenolide standards, digitoxin and digitoxigenin, were spotted in five channels on each TLC plate. $R_{digitoxin}$ values of sample cardenolides were calculated by dividing the migration distance of sample cardenolides by the migration distance of the nearest digitoxin standard, measured to the nearest 0.05 mm.

Rearing Queens on Milkweeds

We induced three *D. gilippus* females to oviposit on *Asclepias curassavica* L. in the laboratory by placing them in silk organza bags that enclosed the stems of the host plants. We transferred the eggs to plastic rearing cups and supplied each emerging larva with fresh foliage from one of the eight milkweed species that queen larvae used in our study area, as well as *A. curassavica* cultivated in our garden. The lab temperature was set at 20°C, and 12 hr of light were provided each day by fluorescent lights that supplemented sunlight coming through windows. Reared adults were frozen approximately 24 hr after eclosion.

Analysis of Thin-Layer Chromatography Data to Determine Host Plants Used by Wild-Caught Queens

Kodachrome 25 images of the TLC plates were taken with a 60-mm macro lens and a Honeywell Prox-o-lite 7 flash system, and then digitized to a Kodak Photo CD by the Digix Corporation (1450 Research Blvd., Suite 200, Rockville, Maryland). The digitized images (resolution = 72 dpi) were viewed and edited on a Power Macintosh 6100/60AV computer with Adobe Photoshop version 3.0 software (Adobe Systems, Inc., 1994). Individual TLC channels were copied from several of the digitized images and pasted onto new files by procedures developed by Gibbs and Brower (in preparation); this allowed us to compare channels more effectively and to produce more informative figures. Brightness and contrast of images were adjusted to better distinguish the blue cardenolide spots from other colored spots. Interplate variation in the distance traveled by cardenolides was corrected for by using the Adobe Photoshop scaling tool, which Gibbs and Brower determined does not alter the $R_{digitoxin}$ values significantly.

Each unique cardenolide fingerprint of the wild-caught butterflies was assigned a number, thus building a reference library of wild TLC patterns. We next assigned letters to the respective cardenolide fingerprints of the queens we

had reared on known host plants. By matching the wild queen numbers with the respective letters of reared queens, we determined their probable host plants. Because many of the wild queens possessed weak cardenolide fingerprints, we split our host-plant assignments into those of high and moderate confidence.

Statistical Analyses With Butterfly Cardenolide Concentration as Response Variable

Because individual queens were proper replicates of each site and each month, we used their cardenolide concentrations directly in statistical analyses of intersite and temporal comparisons. Values were positively skewed at all sites and required nonparametric statistical Kruskal-Wallis or Wilcoxon Mann-Whitney tests.

For interecozone comparisons, we did not use the cardenolide concentration of individual butterflies as our response variable. Instead, we calculated the mean cardenolide concentration of each sample (a sample is defined as the set of queen butterflies captured at a single site on a single day), and looked for treatment effects upon these sample means. Although this approach reduced our sample sizes to as few as three or four means per ecozone, it was necessary in order to avoid pseudoreplication (Hurlbert, 1984). Because the central limit theorem states that the distribution of sample means tends toward normality (Zar, 1974), we performed parametric tests (*t* test and ANOVA) to compare the means of different regions. When variances differed between data sets, log or square root transformations were made.

Statistical Analyses of TLC Patterns in Wild Queens

For statistical analyses of host-plant use, we considered only host-plant assignments of high confidence, i.e., cardenolide fingerprints that had very bold spotting patterns. Data from host plant assignments of high to moderate confidence are given in Table 7 below.

When comparing host-plant utilization of queens among sites, individual queens were proper replicates of the treatment site. Chi-square tests were used for these comparisons and also for analyzing temporal effects upon host-plant use. For interecozone comparisons, we calculated the proportion of wild queens in each sample that possessed the cardenolide fingerprint of *Asclepias perennis/ A. longifolia*. Parametric statistical analyses were performed with the arcsine of the square root of each proportion as the transformed response variable (Zar, 1974). Hurlbert (1984) advocated using parametric analyses for proportion data as a way to avoid pseudoreplication. Lastly, correlation analysis was performed to determine the relationship between the mean cardenolide concentration of each sample and the percentage of queens in each sample that were utilizing cardenolide-rich milkweeds.

Statistical analyses were performed on a Power Macintosh 6100/60 AV computer with Statview 4.01 (Abacus Concepts, Inc., 1992).

RESULTS

Cardenolide Concentrations of Wild Queens

Comparison of Male and Female Queens. In agreement with Cohen (1985) and Ritland (1994), we found no significant differences in cardenolide concentrations between the sexes (Table 3). We therefore pooled data from males and females for subsequent analyses.

Intersite Variation. Within the coastal ecozone (Figures 2 and 3), mean cardenolide concentrations of queens from sites 2, 3, and 4 did not differ significantly, but were all significantly higher than queens from site 1 (Kruskal-Wallis test, $H = 12.94$, $P = 0.0048$). No significant differences were found among sites within the hydric inland ecozone ($H = 5.51$, $P = 0.14$) or among sites within the xeric inland ecozone ($H = 2.64$; $P = 0.27$).

Interecozone Variation. When including data from the entire season of collecting, the frequency distributions of cardenolide concentrations differed greatly among the three ecozones (Figure 4). In the coastal ecozone, the distribution was skewed positively, with 76% (106 of 140) of the queens having concentrations between 0 and 40 μg/0.1 g dry weight. In the hydric inland ecozone, cardenolide concentrations were more normally distributed. In the xeric inland ecozone, 91% (21 to 23) of the queens had cardenolide concentrations between 0 and 40 μg/0.1 g. The mean cardenolide concentration of queens from the hydric inland ecozone was 4.8–5.7 times greater than the means from the coastal and xeric inland ecozones, respectively.

TABLE 3. COMPARISON OF CARDENOLIDE CONCENTRATIONS OF WILD FEMALE AND MALE QUEENS CAPTURED IN THREE DIFFERENT ECOZONES OF NORTHERN FLORIDA

Ecozone	Gender	Conc. (μg/0.1 g dry wt, Mean ± SD)	N	P^a
Coastal	Female	28.0 ± 47.2	58	0.64
	Male	36.0 ± 59.7	82	
Hydric inland	Female	177.0 ± 140.5	39	0.38
	Male	149.7 ± 134.8	49	
Xeric inland	Female	36.0 ± 96.6	8	0.61
	Male	24.1 ± 58.1	15	

[a] Probabilities are from Mann-Whitney U tests.

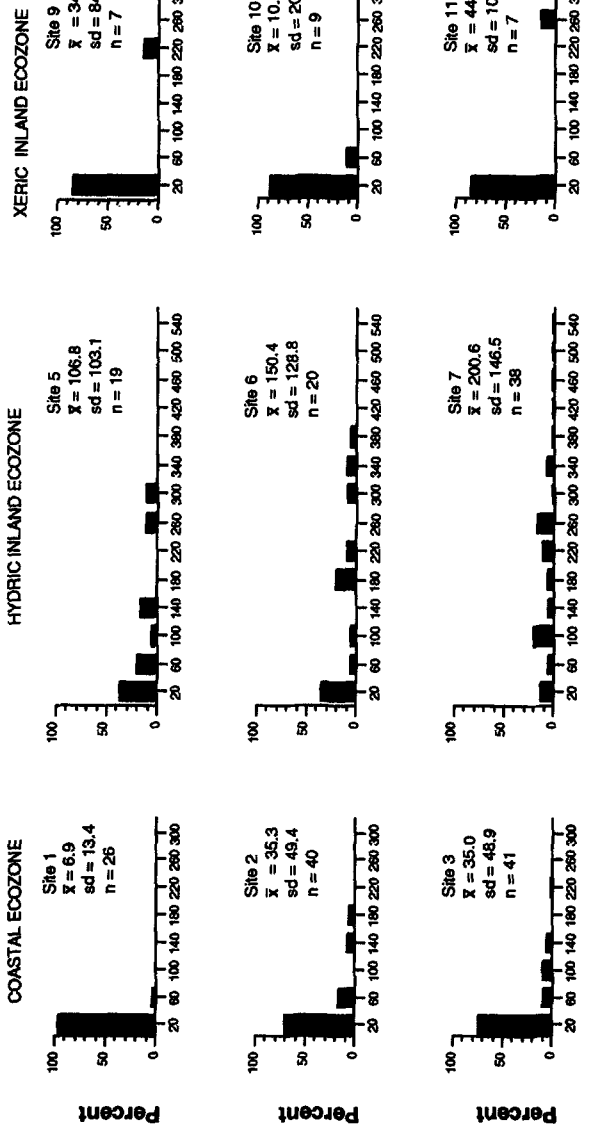

FIG. 2. Frequency distributions of cardenolide concentrations of queen butterflies from the 11 study sites in northern Florida.

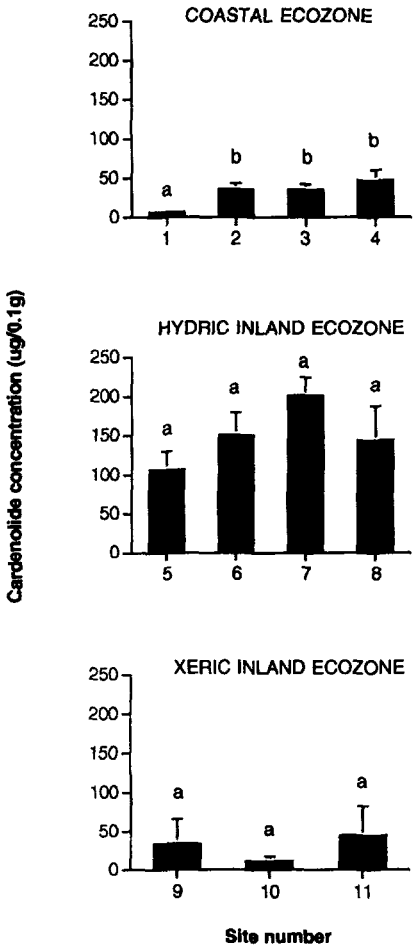

FIG. 3. Intersite variation of cardenolide concentrations of queens captured in three ecozones of northern Florida. Columns indicate mean cardenolide concentrations, plus one standard error. Columns in the same graph that have the same letter are not significantly different. Sample sizes are as in Figure 2.

To preclude month of capture from acting as a confounding factor, a separate statistical analysis comparing queen cardenolide concentrations among ecozones was performed for each month of the study (Table 4). Queens from hydric inland habitats had significantly higher cardenolide concentrations than queens captured on the coast in July (t test, $t = -3.15$, $P = 0.015$) and August ($t = -2.54$, $P = 0.026$). In September, they had higher cardenolide concentrations

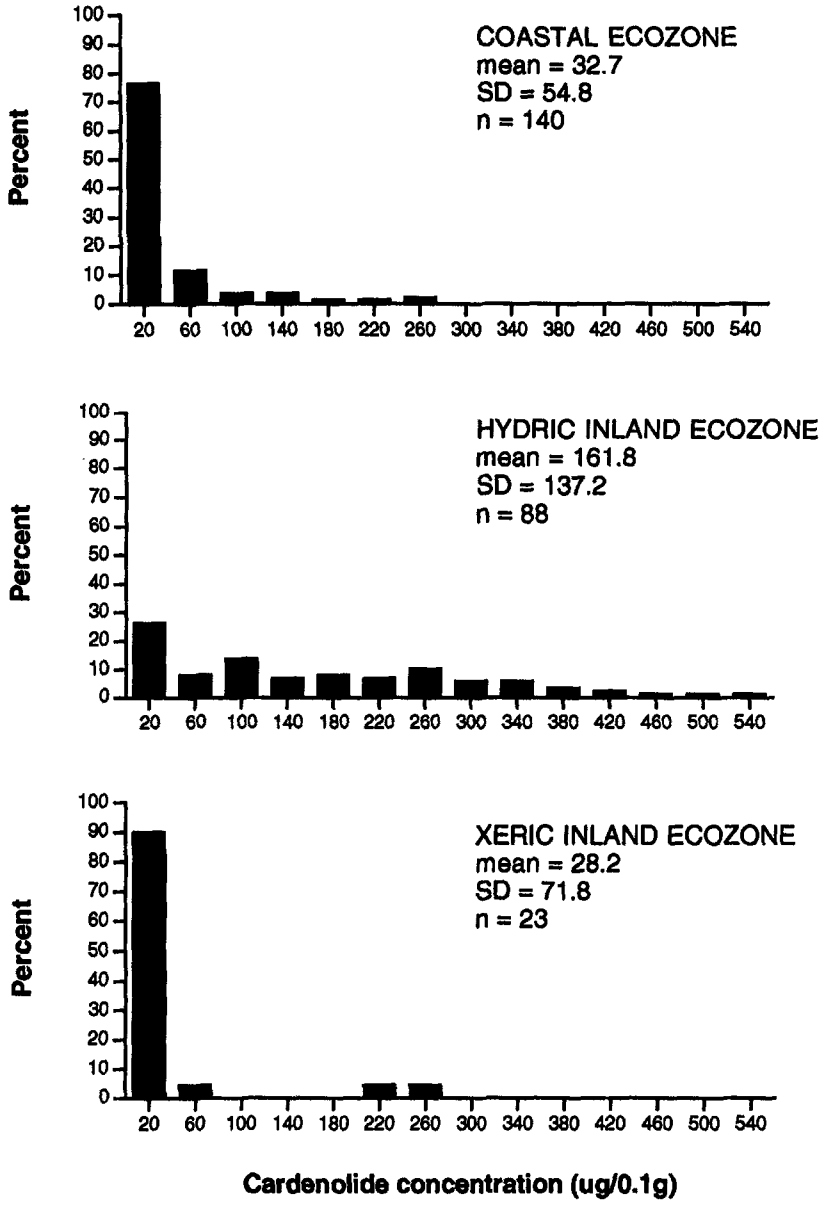

FIG. 4. Frequency distributions of queen cardenolide concentrations from each of the three ecozones in northern Florida.

TABLE 4. MONTHLY COMPARISONS OF CARDENOLIDE CONCENTRATIONS OF QUEEN BUTTERFLY SAMPLES FROM DIFFERENT ECOZONES

Month[a]	Ecozone	Conc. (μg/0.1 g dry wt, mean $\pm$ SD)[a]	N
July	Coastal	$26.7^a \pm 15.0$	4
	Hydric inland	$132.1^b \pm 60.6$	2
August	Coastal	$40.6^a \pm 25.9$	3
	Hydric inland	$142.7^b \pm 83.6$	4
September	Coast	$44.2^a \pm 33.0$	4
	Hydric inland	$208.8^b \pm 54.0$	4
	Xeric inland	$29.9^a \pm 17.2$	3

[a]Means from the same month followed by different letters are significantly different.

than both coastal queens and xeric inland queens (ANOVA, $F_{2,8} = 17.81$, $P = 0.001$; Fisher's protected least significant difference (PLSD) test; $P = 0.001$ and $P = 0.0009$, respectively); coastal queens and xeric inland queens did not differ significantly ($P = 0.63$).

Temporal Variation of Cardenolide Concentration. In the coastal ecozone (Figure 5a), cardenolide concentrations showed a moderate increase over the summer (Kruskal-Wallis test, $H = 12.26$, $P = 0.0065$). *Specifically, queens* captured in August had significantly higher cardenolide concentrations than those captured in July (Mann-Whitney U test, $z = -2.22$, $P = 0.027$), and concentrations higher than those captured in June ($z = -1.94$, $P = 0.053$). Queens captured in September possessed higher concentrations than queens captured in June ($z = -2.649$, $P = 0.0081$) and July ($z = -2.759$, $P = 0.0058$). In the hydric inland ecozone (Figure 5b), the cardenolide concentrations of queens also increased from July to September, but this increase was not statistically significant (Kruskal-Wallis test, $H = 4.51$, $P = 0.10$). We captured queens in the xeric inland ecozone only in September, and therefore could not test for temporal variation within this ecozone (Figure 5c).

Cardenolide Contents of Queen Butterflies Reared on Eleven Species of Northern Florida Milkweeds

Quantitative Analysis. Queens reared in the lab on each of 11 milkweed species had a wide range of cardenolide concentrations (Table 5). The five plant species with high cardenolide concentrations [*Asclepias perennis*, *A. humistrata*, *A. curassavica*, *A. viridis* and *A. longifolia* (Moranz, 1996)] produce queens with high cardenolide concentrations. Queens reared on the other host plants

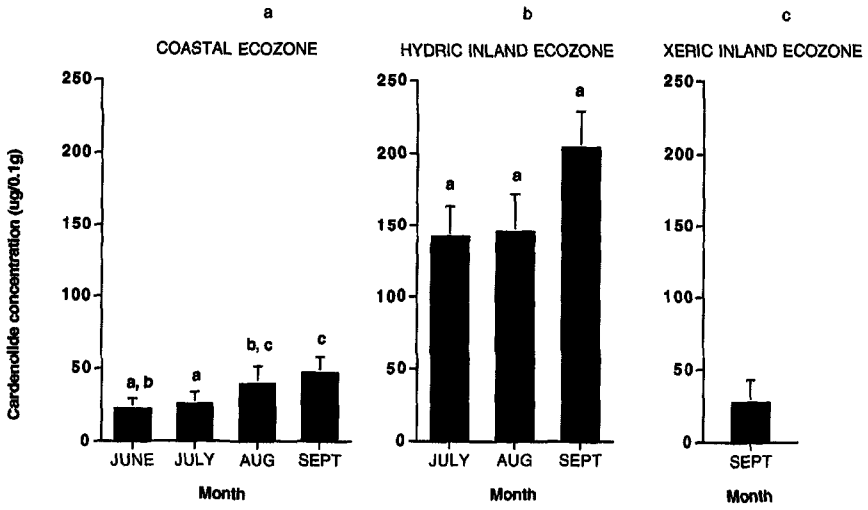

FIG. 5. Mean cardenolide concentrations, plus one standard error, of queen butterflies captured during different months in: (a) the coastal ecozone, (b) the hydric inland ecozone, and (c) the xeric inland ecozone of northern Florida. Columns that share at least one letter are not significantly different; columns that do not share any letters are different at the $P < 0.05$ level. (a) Sample sizes are as follows: June, $N = 40$; July, $N = 39$; August, $N = 28$; September, $N = 33$. (b) Samples sizes are as follows: July, $N = 26$; August, $N = 36$; September, $N = 26$. (c) The sample size is 23.

TABLE 5. CARDENOLIDE CONCENTRATIONS OF QUEEN BUTTERFLIES REARED ON FLORIDA MILKWEEDS

Host species	Conc. (μg/0.1 g dry wt) Mean ± SD	Range	*N*	Florida county Location
Asclepias perennis	377 ± 110	239–485	4	Dixie
Asclepias humistrata[a]	368 ± 58	n.a.	7	Alachua
Asclepias curassavica[b]	336 ± 133	136–757	27	Dade
Asclepias humistrata	327 ± 40	280–363	5	Alachua
Asclepias viridis	256 ± 52	195–334	10	Levy
Asclepias longifolia	250 ± 30	211–283	4	Dixie
Matelea gonocarpa	36		1	Levy
Cynanchum angustifolium	6 ± 8	0–20	9	Levy
Asclepias lanceolata	4 ± 3	0–9	9	Levy
Cynanchum scoparium	3 ± 5	0–12	11	Alachua, Levy
Asclepias tuberosa[b]	1 ± 3	0–12	20	Alachua
Asclepias incarnata[b]	0 ± 1	0–4	13	Dade

[a]Data from: Cohen (1985); queens were reared on foliage collected near Gainesville, Florida.
[b]Data from Ritland (1994); queens were reared on potted plants in Gainesville, Florida.

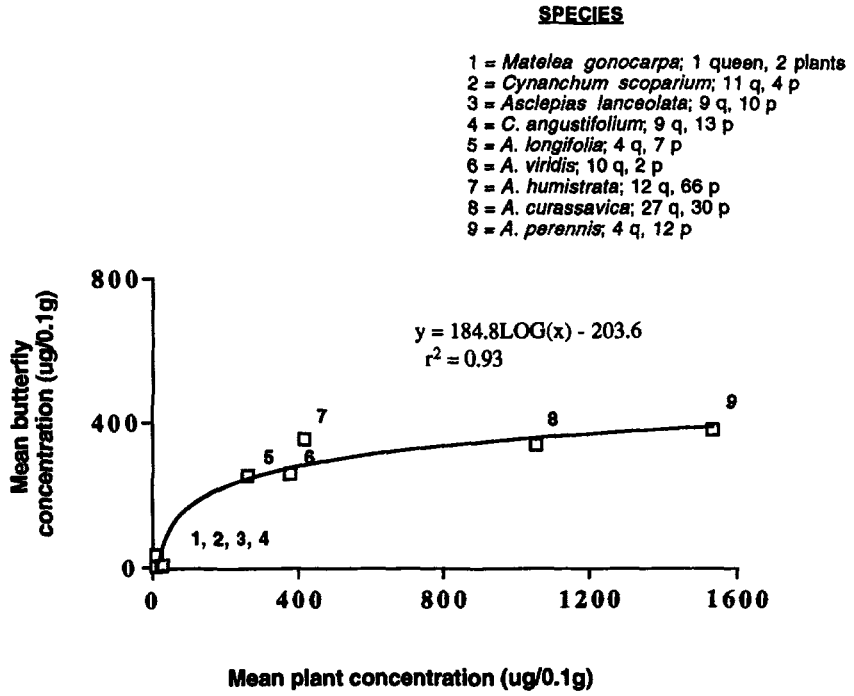

FIG. 6. Logarithmic regression of the mean cardenolide concentrations of 87 queens reared on nine milkweed species over the mean cardenolide concentrations of the leaves of the milkweed species they were reared upon. The queen data are from Table 4. The plant data are from Moranz (1996).

(*Matelea gonocarpa*, *Cynanchum angustifolium*, *A. lanceolata*, *C. scoparium*, *A. tuberosa*, and *A. incarnata*) possess very low concentrations. Regression of the butterfly concentrations on the respective plant concentrations revealed a highly significant, logarithmic relationship (Figure 6). Initially, as plant cardenolide concentration increases, queen cardenolide concentration increases at a high rate, but the rate of increase quickly decreases, especially after the plant cardenolide concentrations reach about 300 μg/0.1 g. This relationship is similar to that previously shown for monarchs and their host plants (Brower et al., 1984; Malcolm et al., 1989).

Qualitative Analysis. Queen and monarch butterflies reared on *A. perennis* and *A. longifolia* have indistinguishable cardenolide fingerprints in the CMF solvent system (Figure 7A). However, their fingerprints are distinguishable from the bold, distinct patterns of queens reared on *A. viridis*, *A. humistrata*, and *A. curassavica* (Figure 7B). The four patterns in Figure 7A and B are distinguishable from one another and from the faint fingerprints of queens reared upon the four cardenolide-poor milkweeds shown in Figure 7C. The fingerprint of the

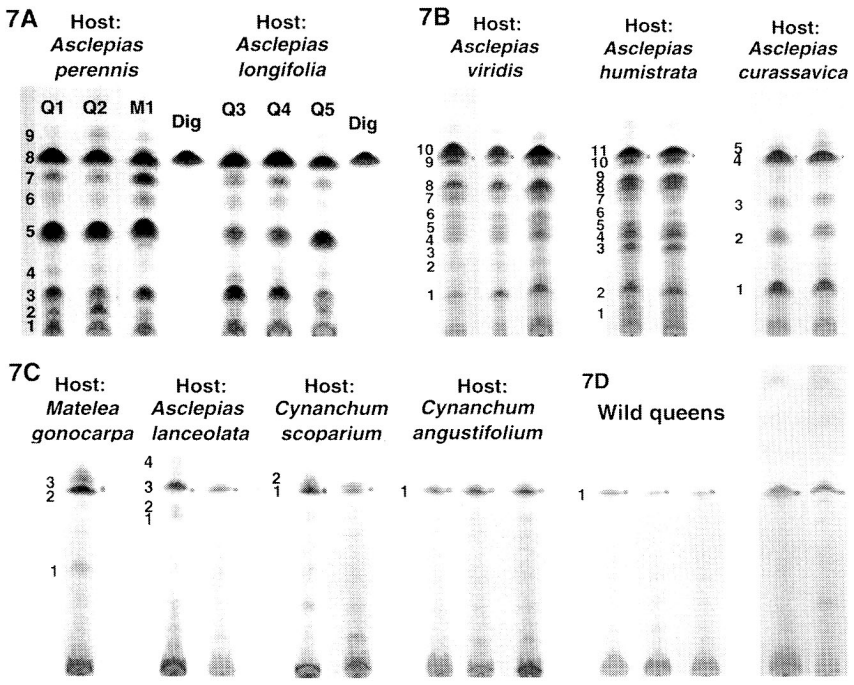

FIG. 7. (A) Thin-layer chromatograms of two queens and one monarch reared on *Asclepias perennis* and three queens reared on *A. longifolia* with 10 μg of digitoxin (Dig) standards. Unlabeled spots are not cardenolides. The channels are from a single plate and are unscaled (see text). (B) Seven queens, each reared on one of three milkweed species: *A. viridis*, *A. humistrata*, and *A. curassavica*. The figure is a composite of channels from three plates. The small black dots near the top of each channel are the scaled positions of the digitoxin standard (see text). (C) Eight queens reared on four cardenolide-poor milkweeds, and three wild queens that presumably fed upon *Cynanchum angustifolium*. The channels are from five plates and were scaled as in B. (D) Two wild queens that exhibit a TLC fingerprint attributed to unknown milkweed 1. The spots are not cardenolides, but provide a distinctive pattern. Channels are from two plates and were scaled as in B.

one queen reared on *Matelea gonocarpa* is distinctive in having a bold spot that migrated beyond digitoxin. Queens reared on *C. scoparium* and *A. lanceolata* were variable, often not exhibiting any cardenolides, sometimes exhibiting a single spot that migrated to the same height as digitoxin, and sometimes exhibiting two or three faint spots. *Cynanchum angustifolium*-reared queens consistently exhibited a single spot that migrated as far as digitoxin.

Spatial Variation of Queen Age

We performed post-hoc analyses to determine if age differences among queens of the three ecozones may have contributed to the variation of cardenolide concentration. Because wing condition deteriorates as butterflies age, we measured wing condition to obtain an approximation of each butterfly's age and compared the wing conditions of queens from the three ecozones. As in our analyses of cardenolide concentration, we used the sample means in our statistical analyses rather than individual measurements, thus avoiding pseudoreplication. The wings of queens from hydric inland habitats (mean wing condition = 2.4, SD = 0.3, $N = 4$) and xeric inland habitats (mean = 2.2, SD = 0.3, $N = 3$) were not significantly different but were in better condition than the wings of coastal queens (mean = 3.2, SD = 0.3, $N = 4$).

Spatial Variation of Host Plant Use by Queens in the Wild

None of the 274 wild-captured queens had the bold, distinctive fingerprints of queens reared upon *A. viridis*, *A. humistrata*, or *A. curassavica*. However, 32% (87 of 274) had a pattern identical to that found in queens reared either on *A. perennis* or *A. longifolia* (Figure 7A), and we had high confidence that these wild queens had fed on one or the other of these two milkweeds. There are six milkweed species in the study area for which we lack cardenolide fingerprints. Of these, *A. cinerea* Walt. is uncommon (Wunderlin, 1982), two (*A. pedicellata* Walt. and *A. tuberosa* L.) are common but cardenolide-poor (Malcolm, 1991), and two (*A. incarnata* L. and *A. verticillata* L.) are both uncommon and cardenolide-poor. The sixth, *Morrenia odorata* (Hook. and Arn.) Lindl., has been recorded only at the southern edges of the study area (Spellman and Gunn, 1976) and is probably cardenolide-poor (as are the other milkweed vines).

When considering only host-plant assignments in which we had high confidence, analyses of TLC data indicated that queens differed in their use of larval host plants among sites in the same ecozone. Twenty percent (24 of 120 queens) from sites 2, 3, and 4 possessed the *A. perennis/A. longifolia* fingerprint, whereas the corresponding value at site 1 was only 3% (1 of 33). This significant difference (Fisher's exact test, $P = 0.0169$) is consistent with the finding (presented earlier) that queens from sites 2, 3, and 4 were significantly more cardenolide-rich than queens from site 1. Correlation analysis provided additional evidence of a relationship between queen cardenolide concentration and host plant use. The mean cardenolide concentration of queen samples was positively correlated with the proportion of each sample that had fed upon *A. perennis* or *A. longifolia* (correlation coefficient $r = 0.873$; $P < 0.0001$; $N = 28$).

Queens in the hydric inland ecozone possessed the *A. perennis/A. longifolia* fingerprint (Table 6) more frequently than queens in the coastal ecozone in July

TABLE 6. MONTHLY COMPARISONS OF MEAN PROPORTIONS OF QUEEN BUTTERFLIES FROM EACH ECOZONE WITH CARDENOLIDE FINGERPRINTS OF *Asclepias perennis* OR *A. longifolia*.

Month	Ecozone	Mean ± SD[a]	N
July	Coastal	$0.095^{a} \pm 0.082$	4
	Hydric inland	$0.638^{b} \pm 0.336$	2
August	Coastal	$0.081^{a} \pm 0.099$	3
	Hydric inland	$0.466^{b} \pm 0.114$	4
September	Coast	$0.327^{a} \pm 0.188$	4
	Hydric inland	$0.782^{b} \pm 0.219$	4
	Xeric inland	$0.085^{a} \pm 0.075$	3

[a]Within each month, proportions with different superscripts are significantly different.

(t test, $t = -3.13$, $P = 0.018$) and August ($t = -4.04$, $P = 0.0034$). In September, the three ecozones differed (ANOVA, $F_{2,8} = 10.77$, $P = 0.0054$). *Asclepias perennis* and/or *A. longifolia* were used more frequently in the hydric inland ecozone than in the coastal ecozone (Fisher's PLSD, $P = 0.017$) and the xeric inland ecozone ($P = 0.0019$). The coastal and xeric inland ecozones did not differ significantly ($P = 0.12$).

Twenty-nine percent (79 of 274) of the wild-caught queens possessed cardenolide fingerprints with a weak spot having the same migration distance as digitoxin (Figure 7C). We determined that these queens had fed upon either *Cynanchum angustifolium*, *C. scoparium*, or *A. lanceolata*, but did so with only moderate confidence, for the fingerprint of the one queen reared on *M. gonocarpa* was similar. Twenty-three queens had cardenolide fingerprints that appeared to be weak forms of the *A. perennis/A. longifolia* fingerprint (Figure 8). We decided with moderate confidence that these queens had fed upon *A. perennis* or *A. longifolia*.

Using host-plant assignments in which we had at least moderate confidence (Table 7), we found that 71% (69 of 97) of the queens captured in hydric inland habitats had fed upon *A. perennis* or *A. longifolia* as larvae. In contrast, only 25% (39 of 153) of the coastal queens had fed upon *A. perennis* or *A. longifolia*, while 39% (59 of 153) of the coastal queens had cardenolide fingerprints similar to those of queens reared on *C. angustifolium/C. scoparium/A. lanceolata*. Queens captured in xeric inland areas usually had very weak cardenolide fingerprints. Only 8% (2 of 24) had the *A. perennis/A. longifolia* fingerprint, while 42% (10 of 24) had a cardenolide fingerprint that matched that of *C. angustifolium/C. scoparium/A. lanceolata*, 17% (4 of 24) had a weak fingerprint that we attributed to a single, unknown host, and 33% (8 of 24) had no detectable cardenolides.

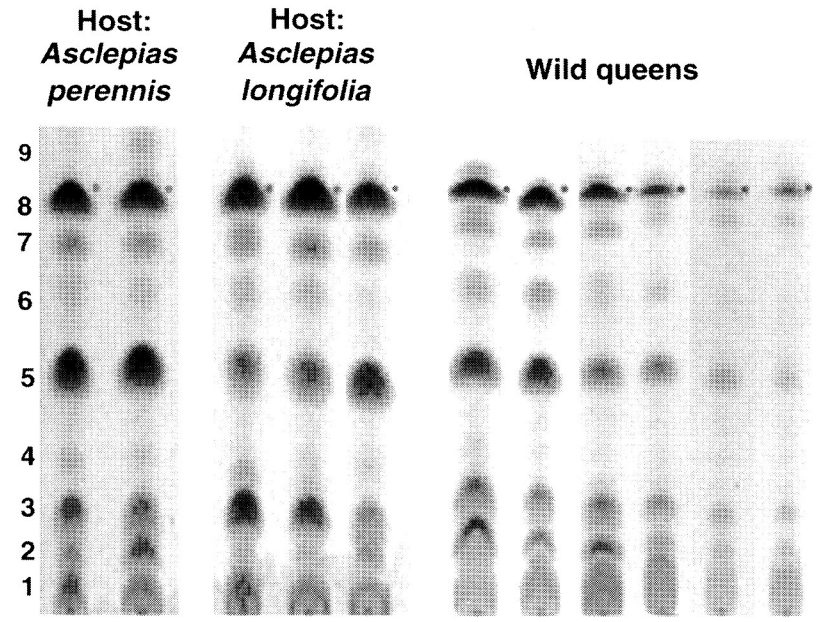

FIG. 8. Queens reared on *Asclepias perennis* and *A. longifolia* are compared to six wild queens that are arranged from high to low cardenolide concentration. The figure is a composite of channels from six plates, scaled as in Figure 7B.

Temporal Variation of Host Plant Use by Wild Queens

Using host-plant assignments in which we had high confidence, we found that 35% (12 of 34) of the coastal queens captured in September possessed the *A. perennis/A. longifolia* fingerprint, significantly higher than the corresponding percentages for June [15% (6 of 40)], July [10% (4 of 41)], and August [8% (3 of 38)] (chi-square test, $\chi^2 = 12.27$, $df = 3$, $P = 0.0065$). A similar trend occurred in the hydric inland ecozone, where 80% (20 of 25) of the queens captured in September had the *A. perennis/A. longifolia* fingerprint, whereas 69% (18 of 26) and 47% (22 of 46) captured in July and August, respectively, had this fingerprint ($\chi^2 = 7.925$, $P = 0.019$).

DISCUSSION

Geographic Variation of Queen Cardenolide Concentration is Host Plant-Mediated

The strong positive correlation ($r = 0.87$) between mean cardenolide concentration of a queen population and the frequency of use of *Asclepias perennis*

TABLE 7. PERCENTAGES OF WILD-CAPTURED QUEENS FROM EACH ECOZONE ASSIGNED TO INDICATED HOST-PLANT SPECIES WITH AT LEAST MODERATE CONFIDENCE

	Ecozone		
Host species	Coastal (%)	Hydric inland (%)	Xeric inland (%)
Asclepias perennis or *A. longifolia*	25	71	8
A. humistrata	0	0	0
A. viridis	0	0	0
Cynanchum angustifolium, *C. scoparium* or *A. lanceolata*	39	10	42
Matelea gonocarpa	1	1	0
Unknown milkweed 1	5	1	17
Indeterminate (weak cardenolide pattern)	14	17	0
Indeterminate (cardenolides absent)	16	0	33
Total percent	100	100	100
Sample Size	153	97	24

and/or *A. longifolia* as determined by TLC strongly suggests that host plants influence the variation of queen cardenolide concentrations in wild populations. Comparing the cardenolide contents of queens in different ecozones showed that those from the hydric inland ecozone of Dixie and Levy counties possessed higher concentrations of cardenolides than queens from either the coastal or xeric inland ecozones. Thin-layer chromatography indicated that a significantly higher proportion of queens in the hydric inland ecozone had fed upon either *Asclepias perennis* or *A. longifolia*. Both of these milkweeds are cardenolide-rich, and engender queen butterflies with high concentrations of cardenolides. It is almost certain that the majority had fed upon *A. perennis*, for it was more common and occupied more habitats. Additionally, the foliage of *A. perennis* is very tender and appears easier for queen larvae to consume than the tough foliage of *A. longifolia*. Most importantly, *A. longifolia* is largely a spring-flowering plant, usually becoming dormant by early summer (Gholsen, personal communication). In contrast, *A. perennis* blooms from April to November, and its foliage is edible to queen larvae throughout this period.

In the hydric inland ecozone, only 26% (23 of 88) of the queens possessed low concentrations of cardenolides (ranging from 0 to 40 μg/0.1 g). It is likely

that *A. lanceolata* was the host to some of these queens, for it appeared to be one of the most common milkweeds in this ecozone, and we frequently found eggs and larvae on the foliage of this plant. In contrast, we did not find any of the milkweed vines (*Cynanchum* spp. or *Matelea* spp.) in the hydric inland ecozone. The TLC data did not provide conclusive evidence because the fingerprint of queens reared on *A. lanceolata* is weak and not consistently distinguishable from those reared on two other cardenolide-poor milkweeds (*C. angustifolium* and *C. scoparium*). An alternative explanation is that these queens had developed along the coast, on the two cardenolide-poor milkweeds just listed, and then dispersed eastward and inland. Dispersal across such a short distance is within the abilities of *D. gilippus*, which expands its breeding range from southern Florida northward to Georgia and the Carolinas in most summers (Burns, 1983), although probably as a result of northward dispersal by successive broods.

Our TLC data indicated that 75% (114 of 153) of the coastal queens had fed upon cardenolide-poor milkweeds. Several factors suggest that *C. angustifolium* is the primary host plant of queens in this ecozone. It is the only milkweed in northern Florida that grows in the coastal salt marshes, an ecosystem that supports large queen populations. We found queen populations wherever we found large patches of this milkweed and observed larvae using this plant at each of our four coastal sites. Burns (1983) also found queens using this species along the Gulf and Atlantic coasts of the southern United States. Analyses of TLC patterns also suggest that a large percentage of coastal queens had fed upon *C. angustifolium*. However, our TLC data did not provide conclusive evidence, due to the similarities among the cardenolide fingerprints of queens reared on *C. angustifolium*, *C. scoparium*, and *A. lanceolata*.

Twenty-five percent (39 to 153) of the coastal queens were cardenolide-rich and possessed the cardenolide fingerprint of either *A. perennis* or *A. longifolia*, but most likely *A. perennis*, for the reasons stated earlier. We hypothesize that these queens had fed upon *A. perennis* in the hydric inland ecozone, then dispersed westward into the coastal salt marshes.

It was surprising to discover that 92% (22 of 24) of the queens captured in xeric inland habitats were cardenolide-poor. Although it might be expected that many queens from the three xeric inland sites would have fed on the cardenolide-rich *A. humistrata*, a known host of *D. gilippus* (Brower 1961, 1962) that occurs commonly in this ecozone, TLC indicated that none had. Two hypotheses could explain this. First, the queens may have fed on the cardenolide-poor *A. tuberosa*, a common plant in the xeric inland ecozone. Second, some queens may have dispersed inland from the Gulf coast, where they had fed on cardenolide-poor milkweeds such as *C. angustifolium* or *C. scoparium*, or from the hydric inland ecozone, where they had fed upon *A. lanceolata*. Forty-two percent (10 to 24) of the xeric inland queens possessed cardenolide fingerprints

resembling those of queens reared on these cardenolide-poor plants, supporting the second hypothesis.

Alternative Explanations for Geographic Variation of Cardenolide Concentrations

Here we present four alternative explanations for our findings and discuss why they are inferior to the hypothesis that host plants caused geographic variation of cardenolide concentrations. First, it was conceivable that the observed differences in cardenolide concentration among coastal, hydric inland, and xeric inland populations were due to age differences in the butterflies. Alonso-Mejia (1991) demonstrated that the cardenolide concentrations of the monarch butterfly ([*Danaus plexippus* L. (Nymphalidae: Danainae)] decrease as the adult monarchs age, possibly due to cardenolide denaturation, loss of scales that contain cardenolides, or loss through physiological processes (Brower et al., 1988; Alonso-Mejia and Brower, 1994; Alonso-Mejia, 1996). Our findings do show differences in the ages of queens, but these trends are incompatible with the interecozone variation in cardenolide concentration, indicating that queen age is not responsible for the differences in cardenolide concentration among the ecozones.

A second alternative hypothesis that may explain geographic differences of cardenolide chemistry is differential selective pressure from predators (Brower, 1984). For instance, predation in hydric inland habitats may be so intense that cardenolide-poor queens are quickly consumed, leaving only cardenolide-rich queens that predators have rejected. This hypothesis assumes that predators are able to reject cardenolide-rich prey based on taste and that the predators release distasteful prey unharmed. Because Fink and Brower (1981) found evidence supporting the first assumption in their studies of the monarch butterfly, this assumption is plausible for the queen butterfly. Regarding the second assumption, if birds, often cited as significant predators of butterflies, do release cardenolide-rich, adult queens unharmed, they almost surely would leave beak marks on the wings (Marshall and Poulton, 1902; Brower, 1984). We carefully inspected all 280 wild-captured queens in our study, and we found no beak marks. Our data therefore do not support the hypothesis of differential selection due to bird predation upon adult queens. It is possible that differential predation upon queen eggs, larvae, and pupae caused the geographic differences in cardenolide chemistry detected in adult queens. However, when considering the immature stages, the second assumption of the differential selection pressure hypothesis is not met: immature queens are soft-bodied and unlikely to survive taste rejection.

A third hypothesis is that genetic variation among queen populations in their ability to sequester cardenolides was responsible for variation of carden-

olide concentration. Eggenberger and Rowell-Rahier (1991) found that interpopulational variation of the autogenous cardenolide defenses of an alpine leaf beetle *Oreina gloriosa* (Coleoptera: Chrysomelidae) was due in part to genetic variation among populations. We cannot rule out the possibility of genetic influence in our study. However, breeding populations of mobile queens in flat, northern Florida are less likely to be genetically distinct than populations of the relatively immobile *O. gloriosa*, which are separated by high Alpine ridges (Eggenberger and Rowell-Rahier, 1991).

Finally, a fourth hypothesis is that abiotic environmental factors are responsible for the variation that we observed, as was suggested for the chemical defenses of *Oreina* leaf beetles (Eggenberger and Rowell-Rahier, 1991). During the summer, the air temperature in the coastal ecozone of our study area is almost 1°C cooler than in the two inland ecozones (Winsberg, 1990). However, it is difficult to see how this or any other climatic difference would lead to low cardenolide concentrations in the disjunct and very different coastal and xeric inland ecozones, yet high cardenolide concentrations in the hydric inland ecozone that is located between them.

After considering abiotic factors and the other three alternative hypotheses, we are confident that host-plant use is the main proximate cause of geographic variation of queen cardenolide concentration in our study area.

Temporal Variation of Queen Cardenolide Concentration

We predicted that queen cardenolide concentrations would change from one brood to the next, due to changing availability of host-plant species that differ in their cardenolide chemistries. Cardenolide concentrations in queens of the coastal habitats did increase significantly from early to late summer, probably due to an increase in the proportion that ate *A. perennis*. Thin-layer chromatography indicated that a higher porportion of queens captured in September used this milkweed than queens captured in June, July, and August. This in turn may have resulted from an increase in immigration of queens reared on *A. perennis* from the hydric inland ecozone, and/or a decrease in the number of queens reared on coastal milkweeds. If a shift in host-plant use did occur, we propose that it was due to the changing suitability of the coastal milkweeds. *Cynanchum angustifolium*, probably the most important coastal host plant, is available for larval development during much of the spring and summer. The same is true for *C. scoparium* and *Matelea gonocarpa*, two other cardenolide-poor milkweed species that are common in coastal habitats. However, the foliage of all three of these milkweeds withers in late summer, while the foliage of *A. perennis* remains in fine condition (Moranz and Brower, pers. obs.). In August and September, queen females may shift their ovipositional preference from *C. angustifolium* and the other cardenolide-poor milkweeds to *A. perennis*, due to the apparent shift in relative host-plant quality.

Implications of Spatiotemporal Variation of Cardenolide Concentration to Palatability

Because cardenolide-rich queens are more unpalatable to birds than cardenolide-poor queens (Ritland, 1994), our discovery that queen cardenolide concentrations vary at the local level supports Ritland's hypothesis that cardenolide-based unpalatability of queens varies among sites separated by only a few kilometers. Forty-nine percent (43 of 88) of the wild queens from the hydric inland ecozone possessed cardenolide concentrations in the same high range as queens that were reared on *A. curassavica* (queens reared on *A. curassavica* are highly unpalatable to birds) (Ritland, 1994). From this, we inferred that many queens in the hydric inland ecozone of northern Florida were highly unpalatable to birds. In contrast, only 6% (9 of 140) and 9% (2 of 23) of queens in the coastal and xeric inland ecozones, respectively, possessed such high concentrations. Additionally, our findings suggest that the palatability of queens decreased from one generation to the next, especially in the coastal ecozone.

Implications of Spatiotemporal Variation of Cardenolide Concentration to Mimicry

Automimicry, the protection of palatable individuals provided by their resemblance to unpalatable conspecifics (Brower et al., 1967; Guilford, 1994), is strongly influenced by variation of the chemical defenses that cause unpalatability (Ritland, 1994). Our study revealed extensive variation in the cardenolide concentrations of queen butterflies within a single site. Therefore, automimicry must occur at the intrapopulation level in northern Florida. From our discovery of interecozone variation of queen cardenolide concentration, we infer that automimicry also occurs among queens occupying different habitats. If avian predators have tasted and/or consumed unpalatable queens in the hydric inland ecozone, they are likely to shun palatable queens when they forage in the coastal and xeric inland ecozones. Finally, temporal variation of the cardenolide concentrations of queens (due to feeding on different milkweeds in successive generations) can also cause automimicry (Ritland, 1994). In northern Florida, predators may consume many palatable queens in June, but in September, they probably encounter a higher number of unpalatable queens, due to the increasing importance of cardenolide-rich *A. perennis* as a host plant.

In his study of the mimetic relationship between queens and viceroys in peninsular Florida, Ritland (1991a) demonstrated that previous conclusions on this classic case of interspecific mimicry were too general as to the direction of the benefit provided by mimetic resemblance. In southeastern Florida, Ritland found that queens were highly unpalatable models and viceroys were moderately unpalatable, resulting in asymmetrical Müllerian mimicry, with queens as the stronger models. He found a different mimetic relationship only 85 km away in

an area of southwestern Florida, where palatable queens were Batesian mimics of unpalatable viceroys. Ritland (1991a, 1994) hypothesized that variation in the mimetic relationship between queens and viceroys must occur elsewhere in Florida. Our data support this hypothesis, and indicate that this variation also occurs on a local scale, even among neighboring ecozones. We suggest that queens and viceroys are distasteful Müllerian mimics of one another at hydric inland sites in Dixie and Levy counties, Florida, while at coastal sites only 5–10 km away, queens probably serve as the palatable Batesian mimics of viceroys.

CONCLUSIONS

This study provides definitive evidence that queen butterflies vary in cardenolide concentrations over short distances and through time. The microgeographic variation is definitively related to the different host plants present in ecologically distinct, neighboring areas. Temporal variation of cardenolides in queen butterflies in the coastal ecozone is related to the increasing usage of cardenolide-rich host plants through time. These findings indicate that the palatability of queen butterflies can vary spatiotemporally, which in turn implies highly dynamic variation in queen automimicry as well as the mimetic relationships between queens and viceroys.

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3-HEXYL-5-METHYLINDOLIZIDINE ISOMERS FROM THIEF ANTS, *Solenopsis (Diplorhoptrum)* SPECIES

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Abstract—The venom alkaloids from the workers of nine collections of *Solenopsis (Diplorhoptrum)* from California contain either (5*E*,9*E*)-3-hexyl-5-methylindolizidine (**1c**) or (5*Z*,9*E*)-3-hexyl-5-methylindolizidine (**1d**) along with *cis*-2-methyl-6-nonylpiperidine. The structures of these compounds were determined from their mass spectra and by comparison of their GC-FTIR spectra with those of a synthetic mixture. In view of the facts that a third diastereomer of 3-hexyl-5-methylindolizidine had been reported in previous collections of *Solenopsis (Diplorhoptrum)* queens from Puerto Rico, and that indolizidines along with other ant venom alkaloids are sequestered by amphibians, the determination of species in this difficult group of ants is significant. In particular, the chemotaxonomic value of the stereochemistry of these venom alkaloids is discussed.

Key Words—Venom, alkaloids, *Solenopsis (Diplorhoptrum)*, indolizidine, chemotaxonomy.

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INTRODUCTION

The 3,5-dialkylindolizidines are one of the earliest reported classes of saturated heterocycles identified in the venoms of ant species in the myrmicine genera *Monomorium* and *Solenopsis* (Ritter et al., 1973; Jones et al., 1984, 1990, 1996). In *Solenopsis* these compounds are found in the "subgenus" *Diplorhoptrum*, a large, worldwide group (Bolton, 1987). Although *Diplorhoptrum* has been elevated to the status of genus (Baroni-Urbani, 1968), it is usually treated as a synonym of *Solenopsis* (Ettershank, 1966). Herein we use *Diplorhoptrum* as a matter of convenience without implying that it is a valid taxonomic entity.

The numerous species of so-called thief ants belonging to *Diplorhoptrum* present many vexing problems for the systematist. There are about 100 names applied to various taxa in the New World, mostly in South America. In the Old World there are almost as many more. Most of these have been superficially described from limited material and, almost invariably, in a nonrevisionary context.

Creighton (1950) recognized about a dozen presumed valid taxa in North America and presented a key for their separation. His key is, unfortunately, largely unworkable, principally due to the unsuspected existence of a number of then undescribed species. This deficiency has been partially rectified by the subsequent recognition and description of a number of these additional species (MacKay and Vinson, 1989; Thompson, 1989; Thompson and Johnson, 1989).

In particular, the vexatious problem of the identity of *S. molesta* Say remains to be resolved. The species level taxonomy of this group is presently unresolved and is likely to remain so until the identity of *S. molesta* is firmly established. This species was originally described by the pioneer American naturalist Thomas Say (1836) from specimens collected at his home in Indiana; none of these original specimens are known to exist in any collection, hence the uncertainty as to the correct identity of this ant. Say's original description (1836) of *S. molesta* is sufficiently vague that it may be applied to any *Diplorhoptrum* in the United States. Since no original material is known to exist, there is no certainty as to which species Say had before him. Our interpretation is that exemplified by Creighton's characterization; even that interpretation is probably polytypic. The venom chemistry of *Diplorhoptrum* shows promise as an aid in the resolution of some of the difficulties of this group.

Indolizidines are also a major heterocyclic class found in the skins of dendrobatid frogs; for example, three isomers of 3-butyl-5-methylindolizidine have been found in an Argentine toad (Garraffo, et al., 1993). There is growing support for the hypothesis that alkaloid-containing amphibians sequester such compounds from their diet (Jones et al., 1996, and references therein), which contains a high proportion of small ants. The ant–frog connection adds to the

importance of the taxonomy of this ant group because it is possible that not all *Diplorhoptrum* may be a source for frog-sequestered compounds. It is not known whether the frogs can discriminate against species that may not be suitable.

There are four possible diastereomers of any 3-alkyl-5-methylindolizidine, and within a particular myrmicine species the structure and stereochemistry of the alkaloids present has not been observed to vary. Previously, in those *Solenopsis (Diplorhoptrum)* species where indolizidines have been found, the natural compounds were the all-*cis* 5*Z*,9*Z* isomer with no trace of the other isomers present; (5*Z*,9*Z*)-3-hexyl-5-methylindolizidine (**1a**) has been detected in collections from Florida and Puerto Rico (Jones et al., 1984, 1996). This report describes the identification of two of the remaining three diastereomers of **1** from the extracts of *Solenopsis (Diplorhoptrum)* species (*S. molesta* group) collected in North America. The implications of different indolizidine diastereomers in separate collections of these ants are presented.

METHODS AND MATERIALS

Chemical Analyses

A Hewlett-Packard model 5890 gas chromatograph equipped with a Rtx-5 30-m × 0.32-mm column was used in gas chromatographic analysis. Vapor-phase FTIR spectra were obtained from a Hewlett-Packard model 5965B detector interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a 30-m × 0.25-mm Rtx-5 Amine column. FTIR spectra of neat liquids were obtained with a Perkin-Elmer 1600 series FTIR instrument. Mass spectra were obtained in the EI mode from a Shimadzu QP-5000 GC/MS equipped with a Rtx-5, 30-m × 0.32-mm column. For high-resolution mass spectrometry (HR-MS) we used a Jeol SX102 instrument equipped with a 15-m × 0.20-mm HP-5 column. Boiling points were uncorrected.

Ants

Solenopsis (Diplorhoptrum) specimens were collected at the sites indicated in Table 1 and immediately placed in small vials of methylene chloride. Voucher specimens of all samples were deposited in the collection of the Los Angeles County Museum of Natural History, Los Angeles, California. The mass spectra and the gas chromatographic retention times of the alkaloids detected in the ants were identical to those of authentic samples. The GC-FTIR spectra of the indolizidines found in collections 1–5 were identical to those of the synthetic isomers prepared below. In collections 3 and 5 the major component had a mass spectrum and gas chromatographic retention time identical to an authentic sample of *cis*-2-methyl-6-nonylpiperidine (Jones et al., 1996).

TABLE 1. 3-HEXYL-5-METHYLINDOLIZIDINES IN *Solenopsis* (*Diplorhoptrum*) SPECIES

Collection	Alkaloids				Comments
	1a	1b	1c	1d	
1 Desert Center, CA			×		
2 Long Beach, CA			×		*cis*-2-methyl-6-nonylpiperidine (t)[a]
3 Alta Loma, CA	t		×		*cis*-2-methyl-6-nonylpiperidine
4 Lake Solano, CA				×	*cis*-2-methyl-6-nonylpiperidine (t)
5 Orange Co., CA	t			×	*cis*-2-methyl-6-nonylpiperidine
6 Irwindale, CA, 1	t			×	*cis*-2-methyl-6-nonylpiperidine
7 Irwindale, CA, 2	t			×	*cis*-2-methyl-6-nonylpiperidine
8 Irwindale, CA, 3	t			×	*cis*-2-methyl-6-nonylpiperidine
9 Marathon Key, FL	×				(Jones et al., 1984)
10 Mona Island, PR	×				Queens (Jones et al., 1996)
11 Cabo Rojo, PR	×				Queens (Jones et al., 1996)
12 Cabo Rojo, PR	×				Queens
13 Cabo Rojo, PR	×				Alate queens

[a]t = trace detected.

Synthesis of **1a–1d**.

2-Hexyl-2-[2-(6-methylpyridyl)-ethyl]-1,3-dioxolane ***(4)***. A solution of 6.0 g (25.8 mmol) of alcohol **2** (Jones et al., 1984) in 20 ml of methylene chloride was added to 13.7 g of pyridinium dichromate in 40 ml of methylene chloride. Fifteen drops of pyridine and six drops of trifluoroacetic acid were added and the solution was stirred overnight. The mixture was diluted with ether and filtered through a florisil column, and the solvent was removed *in vacuo*, providing 4.7 g of 2-methyl-6-(3-oxononyl)pyridine (**3**) as a viscous liquid. IR (neat) 3067, 1713, 1592, 1578, 1156, 1127 cm^{-1}; MS, *m/z* (rel %) 232 (M^+-1, 1), 218 (1), 176 (11), 163 (5), 149 (5), 148 (67), 121 (15), 120 (100), 107 (9). A solution containing 20 ml of ethylene glycol and 5.6 g (24 mmol) of ketone **3** in 100 ml of benzene was acidified by dropwise addition of concentrated HCl and a small crystal of *p*-toluene sulfonic acid. The mixture was refluxed 4hr with a Dean-Stark trap and, after cooling, was neutralized with aqueous $NaHCO_3$. The aqueous layer was extracted with 3 × 40 ml of diethyl ether, the ether layer was dried over K_2CO_3, and the solvent was removed *in vacuo*. The resulting residue, after dilution with a 1:1 hexane–ether solution was filtered through a Florisil column, and the solvent was removed *in vacuo* to give 5.2 g (19 mmol, 78%) of liquid. IR (neat) 3063, 1592, 1579, 1456, 1156, 1136, 1086, 1049 cm^{-1}; MS, *m/z* (rel %) 276 (M^+-1, 1) 192 (95), 157 (100), 148 (7), 120 (18), 107 (8). HR-MS, calcd for $C_{17}H_{27}NO_2$: 277.2042; observed, 277.2047.

*2-Hexyl-2-[2-(6-methylpiperidyl)-ethyl]-1.3-dioxolane (**5a** and **5b**).* A: Sodium-in-Ethanol Reduction. A solution containing 2.0 g (7.2 mmol) of dioxolane **4** in 70 ml of absolute ethanol was stirred and heated to reflux under a drying tube and ca. 15 g of sodium metal was added in small pieces. After 2 hr the mixture was allowed to cool and was quenched by slowly adding water. The aqueous layer was extracted with 3 × 25 ml of ether. The ether layer was washed with brine and dried over K_2CO_3 and the solvent was removed *in vacuo*, producing 1.9 g (6.7 mmol, 93%) of a liquid. GC-MS analysis of the residue showed the presence of two components in a 5:2 ratio with identical mass spectra. MS, *m/z* (rel %) 282 (M^+-1, 1), 268 (1), 198 (20), 157 (17), 98 (100). The GC-FTIR spectrum of the first eluting isomer (**5a**) had bands at 2803 and 2716 cm^{-1} not present in the second isomer. HR-MS, calcd for $C_{17}H_{33}NO_2$: 283.2511; observed, 283.2520; calcd for $C_{17}H_{32}NO_2$: 282.2433; observed, 282.2448.

B: Catalytic Hydrogenation. A solution of 1.018 g of dioxolane **4** and 0.365 g of 5% Rh-on-alumina catalyst in 50 ml of ethyl acetate was hydrogenated at 3 atm for 4 hr. After filtration, the solvent was removed *in vacuo*. GC-MS analysis of the residue showed the presence of one component with a mass spectrum and retention time identical to that of **5a**.

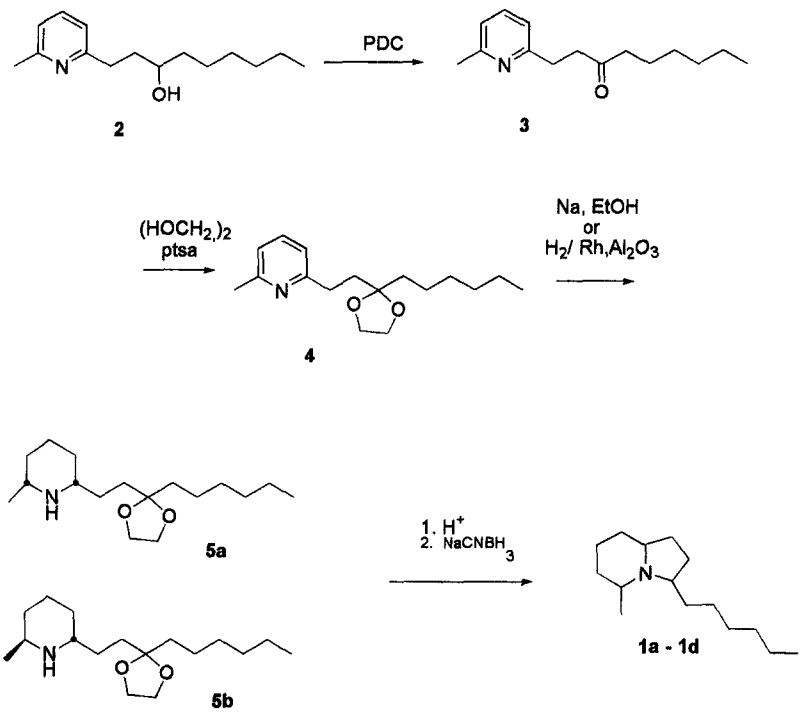
*3-Hexyl-5-methylindolizidine (**1a**, **1b**, **1c**,* and *1d).* A solution containing 1.1 g of the mixture of **5a** and **5b**, 35 ml of THF, 4 ml of water, and 4 ml of concentrated HCl was stirred for 3 hr. The solution was made basic with aqueous NaOH, and the aqueous layer was extracted with 3 × 25 ml of ether. The solvent was removed from the combined ether extracts and the residue was treated with $NaCNBH_3$ as previously described (Jones et al., 1984). The solution was acidified with dilute hydrochloric acid and made basic again with aqueous NaOH. The usual work-up provided 0.7 g (3 mmol, 77%) of a colorless liquid. GC-MS analysis showed four eluting peaks (**1a–1d**) in a 3:2:2:1 ratio with identical mass spectra. MS, *m/z* (rel %) 223 (M^+, 1) 208 (6), 139 (10), 138 (100), 41 (14). HR-MS, calcd for $C_{15}H_{29}N$: 223.2300; observed for first eluting peak, 223.2286; observed for second eluting peak, 223.2297; observed for third eluting peak, 223.2296; observed for fourth eluting peak, 223.2274. A sample of **5a**, from the hydrogenation of **4**, was treated in a similar fashion to provide **1a** and **1c** in 2:1 ratio.

RESULTS AND DISCUSSION

Preliminary GC-MS examination of the extracts of the *Solenopsis* (*Diplorhoptrum*) species in collections 1–8 (Table 1) revealed the presence of 3-hexyl-5-methyl indolizidine (**1**) from its mass spectrum [*m/z* 223 (M^+, 1), 222(1), 139(8), 138(100)]; however, the FTIR spectra of the indolizidines in these collections clearly indicated that they were not the all-*cis* 5*Z*,9*Z* diastereomer

(**1a**) that had been previously described from ants in this genus (Jones et al., 1984, 1996). In previous work the FTIR spectrum showed a broad range of Bohlmann bands, decreasing in intensity from 2800 to 2500 cm^{-1}. In collections 1–3, a single absorption at 2793 cm^{-1} was observed while in collections 4–8 no Bohlmann bands were observed for the hexylmethylindolizidine.

In order to establish the stereochemistry of the indolizidines in these collections, a nonselective synthesis of the four possible isomers was carried out (Scheme 1). The pyridine alcohol (**2**) was successfully oxidized to the ketone (**3**) with pyridinium dichromate. Following ketalization, the pyridine ring was reduced either with sodium-in-ethanol or by hydrogenation over a rhodium catalyst. The former gave a 5:2 mixture of the *cis* and *trans* piperidines **5a** and **5b**, while the latter provided only the *cis* piperidine **5a** (MacConnell et al., 1971). These assignments were confirmed by the FTIR spectra where the spectrum of the *cis*-**5a** showed Bohlmann bands at 2803 and 2716 cm^{-1} that were not present in the spectrum of *trans*-**5b** (Garraffo et al., 1994). The 3-hexyl-5-



SCHEME. 1. Synthesis of the isomeric 3-hexyl-5-methylindolizidines **1a–1d**.

methylindolizidines **1** were formed by reductive amination following removal of the ketal protecting group. Thus, the mixture of **5a** and **5b** provided **1a–1d** in a 3:2:2:1 ratio, while reductive amination of pure **5a** gave only **1a** and **1c**.

Although the isomeric indolizidines **1a–1d** have nearly identical mass spectra, these syntheses along with the FTIR spectra of the four diastereomers (Figure 1) permitted the assignment of stereochemistry for each isomer (Figure 2). In previous studies of 3,5-disubstituted indolizidines, Bohlmann band patterns have been used to assign stereochemistry (Sonnet and Oliver, 1975; Garraffo et al., 1993). The first eluting isomer had been previously prepared stereospecifically (Jones et al., 1984), and its FTIR spectrum matched that of an authentic sample of (5*Z*,9*Z*)-**1a**. The third eluting isomer must be (5*E*,9*E*)-**1c** since it is also formed in the reductive amination of pure *cis*-**5a**.

The second and fourth eluting isomers are those with a *trans*-substituted six-membered ring. In the former, (5*E*,9*Z*)-**1b**, the presence of strong Bohlmann bands indicates the presence of *trans*-antiparallel C–H bonds, and this isomer has been shown to have an axial methyl group configuration (Sonnet et al., 1979; Jones et al., 1984). The last eluting isomer, (5*Z*,9*E*)-**1d**, shows no Bohlmann bands in its FTIR spectrum as a consequence of adapting a *cis*-fused ring system (Figure 2) to relieve the steric crowding that would result from the 1,3-diaxial configuration of the hexyl and methyl groups in a *trans*-fused indolizidine (Sonnet et al., 1979).

The (5*E*,9*E*)-**1c** and (5*Z*,9*E*)-**1d** indolizidines found in collections 1–8 had FTIR spectra and GC retention times that matched those of the synthetic isomers. Their stereochemistry contrasts with the (5*Z*,9*Z*)-**1a** found in collections 9–13, including previous investigations (Table 1) and with the 5*Z*,9*Z* 3-ethyl and 3-butyl indolizidine analogs found in *Solenopsis conjurata* and *Monomorium pharaonis* (Jones et al., 1984; Ritter and Persoons, 1975). Additionally, collections 3 and 5 contained more of the monocyclic homolog, *cis*-2-methyl-6-nonylpiperidine, than indolizidine **1c** or **1d**. The stereochemistry of this piperidine was established by direct comparison with an authentic sample.

The 5*Z*,9*Z* isomer **1a** had previously been found as a caste-specific compound in queens of a *Solenopsis* (*Diplorhoptrum*) species (Jones et al., 1984, 1996). A reinvestigation of a collection from Cabo Rojo, Puerto Rico, has confirmed this and demonstrated its presence in queens and alate queens from the same nest (Table 1). Within a group such as *Diplorhoptrum*, superficially similar species may produce different venom alkaloids or alkaloids in varying combinations. Thus it was shown that the queens of two similar species (collections 10 and 11) both produce indolizidine **1a**. However, their respective workers were sufficiently different chemically that they clearly belong to different taxa despite their morphological similarities: workers of 10 produce an unsaturated piperidine while those of 11 produce two indolizidine 223AB

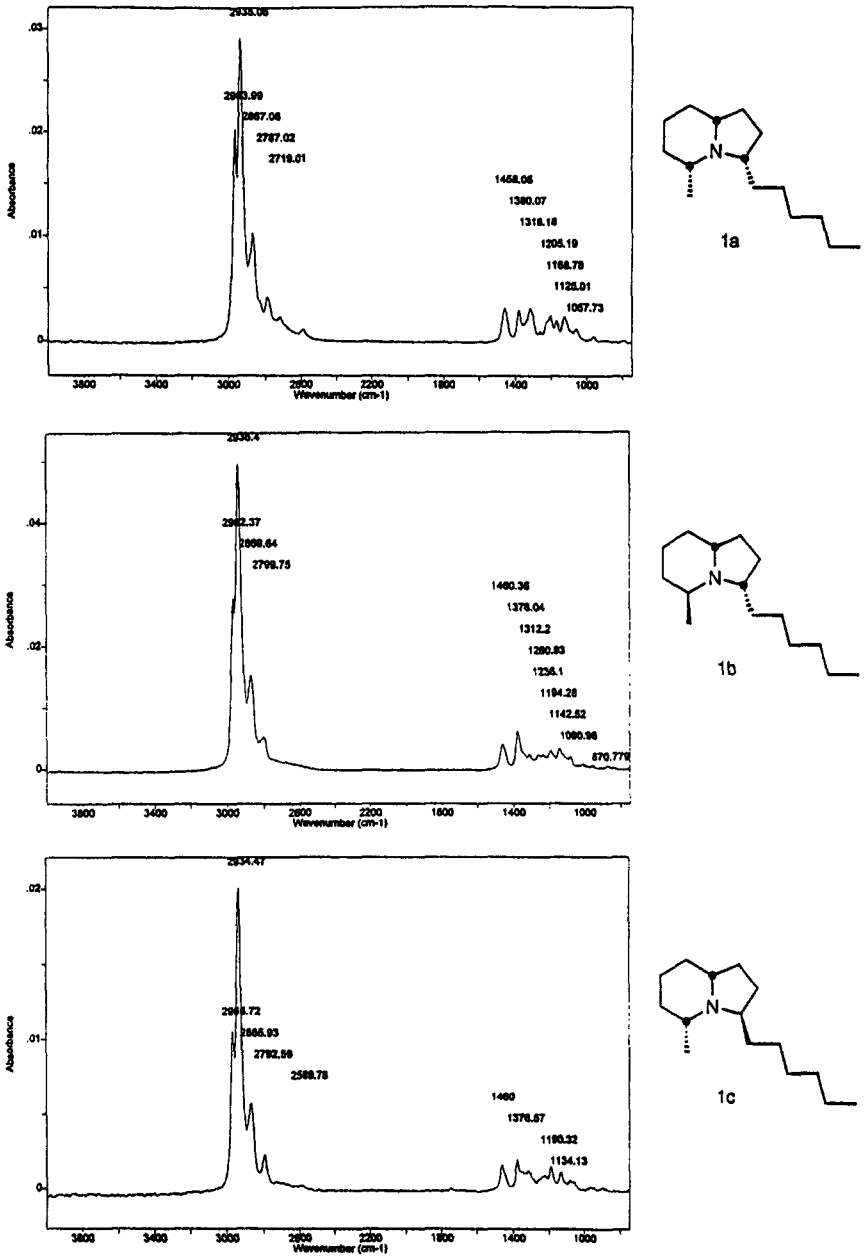


FIG. 1. Vapor-phase FTIR spectra of the indolizidine isomers **1a–1d**.

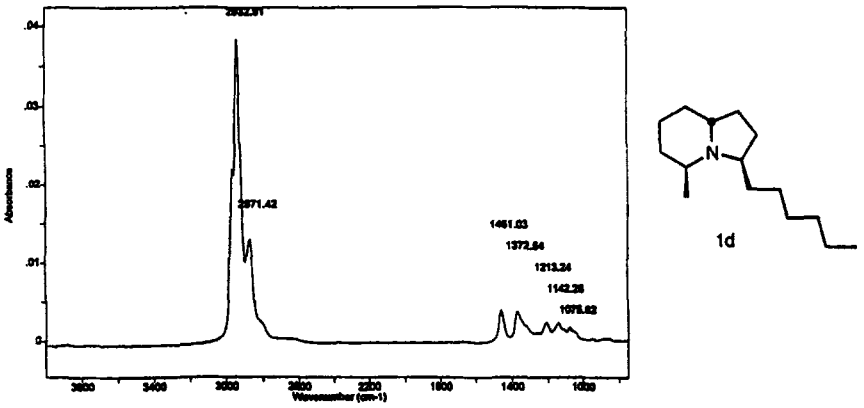


FIG. 1. Continued.

diastereomers (Jones et al., 1996). These *Solenopsis* (*Diplorhoptrum*) containing indolizidine **1a** are clearly different species and castes from those containing indolizidines **1c** or **1d**.

These results showing the occurrence of three of the four diastereomers of 3-hexyl-5-methylindolizidine (**1**) in *Solenopsis* (*Diplorhoptrum*) species naturally raise the question of whether venom alkaloid stereochemistry can play a

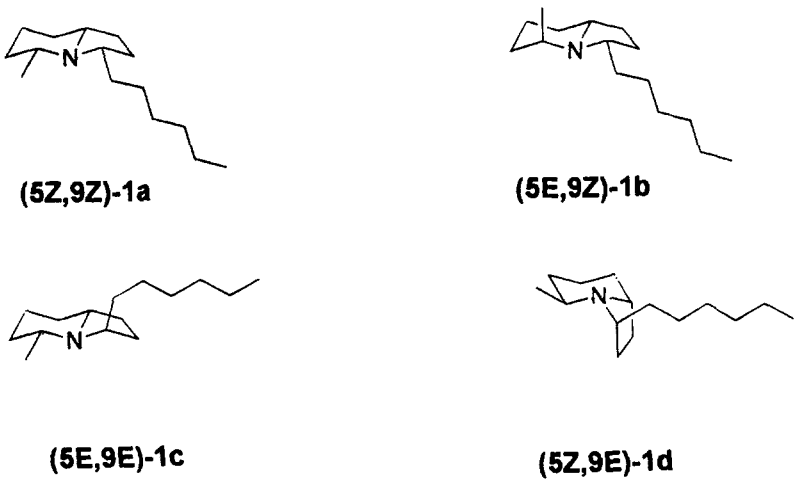


FIG. 2. Four possible diastereomers of 3-hexyl-5-methylindolizidine **1**.

significant role in the taxonomic characterization of these ants. It has been shown that the venoms of *Solenopsis* (*Solenopsis*) species (fire ants) are characterized by 2-alkyl-6-methylpiperidines, compounds that occur as *cis* and *trans* diastereomers, and particular species produce different mixtures of these compounds (Brand et al., 1972). Even when hybrids between species occur, and their venom compositions reflect both species, the ratios of *cis*/*trans* stereoisomers remain unchanged (Vander Meer and Lofgren, 1988). The *Solenopsis* piperidine alkaloids arise from a polyacetate chain, with the ring stereochemistry being established in the final reduction of a piperideine intermediate (Leclercq et al., 1996). If this step is enzymatically controlled, as is likely given the stereospecificity observed in these and other natural ant venom alkaloids, then the stereochemistry of these compounds should be considered to be a valid taxonomic character.

The specimens from California (Table 1) were described as a variety (*validiuscula*) of *S. molesta* by Emery (1895). However, since the venom of collections 1–3 contains **1c** while collections 4–8 contain **1d**, collections 1–3 are probably not conspecific with collections 4–8 even though both fall within the concept of *S. molesta validiuscula* as explicated by Creighton (Creighton, 1950). Which, if either, of the California forms is actually *validiuscula* is unclear, as is the identity of *S. molesta*; we predict that neither is a form of the eastern species.

In the future, the evidence provided by analysis and identification of venom alkaloids may allow the sorting of samples into putative taxonomic units. With such sorted samples available, it may be possible for a systematist to discern features of external morphology such as would resolve some of the current difficulties with the systematics in this taxonomically challenging complex.

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EFFECT OF NITROGEN AVAILABILITY ON EXPRESSION OF CONSTITUTIVE AND INDUCIBLE CHEMICAL DEFENSES IN TOMATO, *Lycopersicon esculentum*

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Abstract—Young tomato plants (*Lycopersicon esculentum*) grown in sand in a greenhouse and subjected to different fertilization regimes were used to test the effects of nitrogen availability on constitutive levels of phenolics and on constitutive and inducible activities of polyphenol oxidase and proteinase inhibitors. Theories that emphasize physiological constraints on the expression of phytochemicals predict an increase in levels of carbon-based allelochemicals under moderate nitrogen stress but predict, under the same conditions, an attenuation of chemical responses involving nitrogen-containing compounds such as proteinase inhibitors and polyphenol oxidase. We found that nitrogen availability had a strong effect on constitutive levels of phenolics; weaker effects on constitutive polyphenol oxidase activity, constitutive proteinase inhibitor activity, and inducible polyphenol oxidase activity; and no effect on inducible proteinase inhibitor activity. These results point to a need for the integration of theories that emphasize physiological influences on secondary metabolism with those that emphasize ecological influences on secondary metabolism and suggest that current theories of plant defense do not adequately account for enzymatic and proteinaceous defenses against arthropods.

Key Words—*Lycopersicon esculentum*, *Helicoverpa zea*, induced resistance, inducible chemistry, phenolics, proteinase inhibitors, polyphenol oxidase, nitrogen availability.

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INTRODUCTION

Although the nature and extent of the evolutionary relationships between plants and herbivores are still disputed (Edwards, 1992), there is little question that the natural products of plants serve to protect them against herbivores and that these metabolites are major, if not primary, determinants of plant resistance to herbivores (Hedin, 1983; Harborne, 1988; Berenbaum and Rosenthal, 1992; Berenbaum, 1995). The levels of these chemicals can vary markedly among plants of the same species and among organs or leaves of the same plant (Denno and McClure, 1983; Karban, 1992). Because of the importance of these chemicals in plant–herbivore interactions, the factors that determine between- and within-plant allocation to secondary chemistry are of interest to ecologists and agricultural scientists.

Physiological constraints imposed by resource availability clearly influence allocation to secondary metabolism in many plants (Coley et al., 1985; Waterman and Mole, 1989; Reichardt et al., 1991; Herms and Mattson, 1992). The quantity and quality of nitrogen available to the plant has received the most attention in this regard and has been shown to influence the levels of a wide variety of types of secondary metabolites, including glucosinolates (Hugentobler and Renwick, 1995), cardenolides (Hugentobler and Renwick, 1995), phenolics (Reichardt et al., 1991; Wilkens et al., 1996b), alkaloids (Baldwin et al., 1993), and furanocoumarins (Zangerl and Berenbaum, 1995). Physiological constraints, however, do not fully explain patterns of allocation to secondary metabolism in most plants (Lerdau et al., 1994; Berenbaum, 1995). Allocation to secondary metabolism is also influenced by ecological demands for defense (e.g., the need to protect valuable or particularly conspicuous tissues or organs). An important factor of this type is the demand for defense that is signaled by herbivore attack (Lerdau et al., 1994). In many plants, feeding by herbivores initiates qualitative and quantitative changes in secondary metabolism (Tallamy and Raupp, 1991; Baldwin, 1994; Karban and Baldwin, 1997), a phenomenon that has been termed induced (in opposition to constitutive) phytochemistry. Induced changes in phytochemistry can extend beyond damaged tissues, are often rapid (occurring in a matter of hours to days), and are often correlated with systemic increases in resistance to subsequent herbivory (Tallamy and Raupp, 1991; Karban and Baldwin, 1997).

Physiological and ecological influences on patterns of allocation to secondary metabolism are not exclusive, although attempts to explain patterns of allocation by integrating physiological and ecological explanations are limited (Berenbaum, 1995). The growth–differentiation balance hypothesis (GDBH), as outlined by Herms and Mattson (1992) and Lerdau et al. (1994), provides a theoretical framework within which it may be possible to integrate physiological and ecological explanations of phytochemical expression. The GDBH argues that expression of phytochemicals is constrained by a negative correlation

between growth and differentiation (secondary metabolism). In resource-rich environments, resources are allocated primarily to growth and resources are diverted away from secondary metabolism. In contrast, when plants are subjected to a stress that limits growth more than it limits photosynthesis, the GDBH predicts increases in allocation to secondary metabolism (ultimately because allocation to defense is favored under conditions of low growth). The GDBH does not exclude the possibility that other ecological factors also influence allocation to secondary metabolism, but such influences are superimposed upon, and must operate within, this physiological constraint (Herms and Mattson, 1992; Lerdau et al., 1994).

In the experiments reported here, we evaluated the separate and interactive effects of damage and nitrogen availability on the constitutive and inducible defenses of tomato (*Lycopersicon esculentum* Mill) foliage. There is reason to hypothesize that rapid induced responses would be sensitive to nitrogen limitation, since many responses involve transcriptional activation of genes and enzymatic activity, and since many inducible molecules—both end products and intermediates involved in signal transduction—contain N (Herms and Mattson, 1992). However, the impact of plant nutrition on rapid chemical induction has been investigated in only a few species of plants (Johnson et al., 1989; Herms and Mattson, 1992; Zangerl and Berenbaum, 1995).

The tomato is a particularly appropriate system for investigating the effects of nitrogen availability on constitutive and inducible secondary metabolism. Resource availability has been shown to influence allocation to both constitutive structural defenses (trichomes) and constitutive chemical defenses (phenolics) in tomato (Wilkens et al., 1996a,b). In addition, minor, localized damage induces at least 12 chemicals in young tomato plants (Schaller and Ryan, 1995) and also induces systemic resistance to noctuids, aphids, leafminers, mites, and the causal agent of bacterial speck (Stout and Duffey, 1996; Stout et al., 1998a). Of the chemical changes that take place following damage, the increases in proteinase inhibitors and the oxidative enzyme polyphenol oxidase appear to be most critical to the induction of resistance (Stout et al., 1988b). Because proteinase inhibitors and polyphenol oxidase are proteins, and because their induction involves changes in transcription and the production of peptide signal molecules (Schaller and Ryan, 1995), induction might be expected to be constrained under low nitrogen conditions in tomato foliage. The extent to which induction is not constrained by nitrogen availability may indicate a priority in allocation given to ecological demands relative to physiological constraints.

METHODS AND MATERIALS

General. Two experiments were conducted to determine the influence of nitrogen availability on constitutive levels and inducibility of tomato foliar

allelochemicals. In the first, plants were subjected to one of three fertilization regimes: low N (maximum N concentration: 2 mM), intermediate N (maximum N concentration: 4 mM), and high N (maximum N concentration: 8 mM). In the second experiment, the intermediate-N treatment was deleted, and plants were subjected to only low-N or high-N regimes. After 25–26 days of growth, assays for total protein and phenolics were performed, and a number of plants in each nutrient treatment were damaged with larvae of the corn earworm (*Helicoverpa zea*) (experiment 1) or sprayed with jasmonic acid (experiment 2). Feeding by *H. zea* and treatment with jasmonic acid have been shown to induce polyphenol oxidase and proteinase inhibitors in young tomato plants (Stout et al., 1996a; Thaler et al., 1996). Comparison of chemical levels and activities in damaged and undamaged plants subjected to different fertilization regimes allowed us to assess the effects of plant nutrition on constitutive and inducible chemistry.

Plant Growth and Fertilization. For both experiments, plants (*L. esculentum* cv. Bonnie Best) were grown from seed in a greenhouse, with natural light supplemented by a single sodium vapor lamp (16L : 8D). Seeds were germinated in vermiculite in flats and watered with distilled water only for 10 days after sowing. On the eleventh day, seedlings were transplanted into 4-in. pots (one seedling per pot) containing sterilized sand, and seedlings were randomly assigned to a low-nitrogen treatment, an intermediate-nitrogen treatment (experiment 1 only), or a high-nitrogen treatment.

Levels of nitrogen received by plants in low-, medium-, and high-N treatment groups were manipulated by fertilizing plants with modified Hoagland's solutions containing different concentrations of N. The nutrient solutions used in these experiments are described in Table 1 (all chemicals were purchased from Sigma, St. Louis, Missouri). Fertilization regimes were chosen to avoid extreme changes in the level of nitrogen given to a plant. Thus, for the first week following transplantation, plants in all treatment groups were fertilized daily with 20 ml of a Hoagland's solution containing 2 mM N. From days 18 to 22, plants in the low-N treatment continued to receive 20 ml of the 2 mM N solution daily, while plants in the high- and intermediate-N treatment were watered with solutions containing 4 mM N. From days 23 to 25, and until experiments were completed, plants in the high-N treatment were given 20 ml of a solution containing 8 mM N; plants in the other treatments received the same solutions they had received on days 18–22. All plants were given additional distilled water as necessary.

Experimental Protocol. Twenty-five days after sowing, plants of all treatments possessed at least three expanded leaves and an expanding fourth leaf. Plants in the high-N treatments were larger than plants in the other treatments: the mean dry mass of plants in the high-N treatment was about twice that of plants in the low-N treatment (data not shown), and plants given higher levels

TABLE 1. MODIFIED HOAGLAND'S SOLUTIONS USED IN EXPERIMENTS 1–2[a]

Compound	Final concentration (mM) in solution		
	Low (2 mM N)	Medium (4 mM N)[b]	High (8 mM N)
$(NH_4)_2SO_4$	0.5	1.0	2.0
$Ca(NO_3)_2$	0.5	1.0	2.0
K_2HPO_4	1.0	1.0	1.0
K_2SO_4	1.0	1.0	1.0
$MgSO_4$	2.0	2.0	2.0
$CaSO_4$	1.5	1.0	
FeEDTA	0.02	0.02	0.02

[a] Solutions contained nitrogen at concentrations of 2, 4, or 8 mM and were prepared according to Blankendaal et al. (1972). All solutions contained 1 ml/liter of a micronutrient solution.
[b] Experiment 1 only.

of N sometimes had four expanded leaves and a fifth expanding leaf. On day 25 or 26, leaflets from six to eight plants per N treatment were harvested for assays of total protein and total phenolics. In experiment one, leaflets for protein and phenolic assays were taken from leaves 1, 3, and 4 (true leaves numbered from the cotyledon, with leaf 1 being the oldest leaf and leaf 4 the youngest). In experiment 2, leaflets for phenolic and protein assays were taken only from the third leaf. Normally, the terminal-most leaflet pair was harvested for these assays; one leaflet of the pair was taken for the phenolic assay and the other for the protein assay.

After assaying plants for phenolics and total protein, the remaining plants in each N treatment were assigned in equal numbers (N = 8–10) to either an induction treatment or a control treatment. Plants in the first experiment were induced by confining a single *H. zea* larva to the terminal leaflet of the third leaf and allowing the larva to feed for 8–12 hr. Plants in the second experiment were induced by spraying the terminal leaflet of the third leaf with a 0.5 mM solution of jasmonic acid until runoff (Thaler et al., 1996). Proteinase inhibitor and polyphenol oxidase activities were determined from undamaged/unsprayed leaflets of the third leaves of both control and induced plants 48 hr after damage/treatment.

Chemical Assays. For the polyphenol oxidase assay, individual leaflets were excised with a razor blade, weighed, and macerated in buffer using a tissue grinder. The extraction buffer (1.25 ml/leaflet) consisted of ice-cold pH 7 K Phos (0.1 M) buffer with 7% polyvinylpolypyrolidine. To this leaf homogenate was added 0.4 ml of 10% Triton X-100. The mixture was vortexed, centrifuged

at 6000*g* for 15 min, and the resulting supernatant used directly for spectrophotometric determination of polyphenol oxidase activity (Stout et al., 1996a). The reaction mixture contained an aliquot (usually 15 μl) of enzyme extract and 1 ml of 2.92 mM caffeic acid in pH 7 K Phos (0.1 M) buffer. Polyphenol oxidase activities were determined by monitoring the rate of formation of melanin-like product from caffeic acid at 470 nm. Polyphenol oxidase activities are presented as change in optical density per minute per gram.

The proteinase inhibitor assay was based on the ability of plant extracts to inhibit chymotryptic activity. Leaf extracts were prepared by grinding leaflets in 50 mM Tris HCl (pH 7.8) buffer (3 ml/g leaf tissue) containing 7% polyvinylpolypyrrolidine, 1.67 mM phenylthiourea, 0.3 M KCl, and 0.4 mM ascorbic acid. This extract was immediately frozen for later use (the proteinase inhibitor activity of an extract is not altered by freezing). For proteinase inhibitor assays, the frozen extract was thawed and centrifuged at 13,000*g*, and 25 μl of the resulting supernatant was added to 25 μl of a 0.001 N solution of HCl containing 0.001 mg of chymotrypsin. This mixture was then allowed to incubate for 10 min. Following incubation, the chymotrypsin/proteinase inhibitor extract mixture was added to 2.9 ml of 0.5 mM benzoyl tyrosine ethyl ester (BTEE, a chymotrypsin substrate) in a methanol–phosphate buffer (0.1 M, pH 8.0) mixture (12 : 13 ratio) and the increase in absorbance at 256 nm recorded for approximately 10 min. A control was run with BTEE and chymotrypsin only. Higher proteinase inhibitor activities are reflected by lower chymotrypsin activities; the relationship between proteinase inhibitor level and chymotrypsin activity is linear for a wide range of proteinase inhibitor levels (Stout et al., 1998a). Proteinase inhibitor activities are reported as percent inhibition of control chymotrypsin activity.

The assay for total protein used the Bradford method as modified for plant protein by Jones et al. (1989). Individual weighed leaflets were homogenized using a tissue grinder in 0 .1 N NaOH (2 ml/leaflet), let sit for 30 min, then centrifuged at 6000*g* for 15 min. An aliquot (8 μl) of supernatant was added in a cuvette to 1 ml Biorad solution with added polyvinylpyrolidine (3 mg/ml) and mixed. Absorbance was read at 595 nm within 20 min. A standard curve was constructed using ribulose 1,5-diphosphate carboxylase-oxygenase; protein levels are expressed as percent protein (wet weight).

For the phenolic assay, individual leaflets were excised with a razor blade, weighed, and immediately frozen by placing them in a freezer. After freezing, leaflets were lyophilized, then placed in 5 ml 50% methanol. Dried leaflet samples in 50% methanol were placed in an oven at 60°C for 24 hr, cooled to room temperature, and brought back to volume before assaying phenolics. Total phenolics were assayed from the methanolic extracts using the Folin-Ciocalteau method (Waterman and Mole, 1994). An aliquot (usually 100 μl) of the methanolic extract was diluted to 2.75 ml with distilled water in a test tube and

vortexed. Folin-Ciocalteau reagent (0.5 ml of a 1 N solution; Sigma) was then added, with mixing, to the diluted plant extract. Within 5 min, 0.5 ml of 20% sodium carbonate was added, the solution vortexed, and allowed to sit for 90 min at room temperature. After 90 min, absorbance of each sample at 720 nm was read. A standard curve was constructed using chlorogenic acid. Total phenolic levels are expressed as micromoles per gram fresh weight.

Data Analyses. Comparison of levels of protein and phenolics among plants of the different nitrogen treatments allowed us to assess the effect of nitrogen level on constitutive levels of these compounds. Data were analyzed by one-way ANOVA or, when multiple leaves were sampled (experiment 1), two-way ANOVA. Data for proteinase inhibitors and polyphenol oxidase were analyzed separately by two-way ANOVA, with damage treatment and nitrogen level as the independent variables. Proteinase inhibitor and polyphenol oxidase data were arc-sine transformed and log-transformed, respectively, before analysis.

RESULTS

Experiment 1. Plants were subjected to one of three fertilization regimes for 25 days, and total protein and phenolics sampled from three leaves on each plant. Half the remaining plants of each fertilization regime were damaged and the other half left undamaged. Two days later, the effects of nitrogen levels on inducibility were assessed by comparing polyphenol oxidase and proteinase inhibitor activities.

Levels of total protein in leaflets were positively correlated with nutrient level and negatively correlated with leaf age (Figure 1, Table 2). In the oldest leaf (leaf 1), protein levels ranged from 1.5% wet wt in plants of the low-N treatment to 2.6% in plants of the high-N treatment; in the third leaf, protein levels ranged from 3.3% (low-N) to 4.1% (high-N); in the youngest leaf, protein levels ranged from 3.8 to 6.1%. Thus, the fertilization regimes used were sufficient to produce differences between treatments in levels of foliar protein (and, assuming total protein to be an index of nitrogen, foliar nitrogen).

Levels of phenolics were also strongly affected by both N treatment and leaf age (Figure 2, Table 2). Leaflets from a given position on plants in the low-N treatment had levels of phenolics more than twice those found in corresponding leaflets from high-N plants, with plants in the intermediate-N treatment having intermediate levels. Phenolic levels were lowest in the oldest leaves (leaf 1) and higher—up to 35% higher—in younger leaves (leaves 3 and 4).

Nitrogen level had no effect on constitutive activities of proteinase inhibitors, which were low in undamaged plants of all three N treatments (Figure 3, Table 2). Moreover, fertilization regime had no effect on the inducibility of proteinase inhibitors. Activities of proteinase inhibitors were equally high in

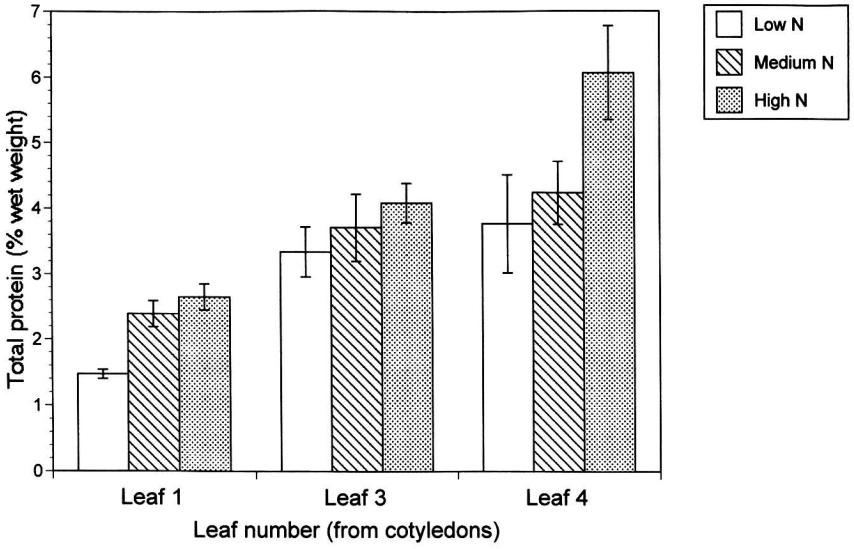

FIG. 1. Effect of nitrogen availability on concentrations of total protein (% wet weight ± 1 SE) at three leaf positions in four-leaf tomato plants. Fertilization regimes are described in the text. Leaf 4 was the youngest leaf; leaf 1 the oldest.

undamaged leaflets of damaged leaves of all three N treatments (Figure 3, Table 2).

Activites of polyphenol oxidase were higher in undamaged leaflets of low-N plants than in undamaged leaflets of high-N plants (Figure 4, Table 2, significant effect of N level on polyphenol oxidase). In addition, damage caused a significant induction of polyphenol oxidase activities (Figure 4, Table 2). There was no significant difference in (absolute) polyphenol oxidase activities in damaged plants of the three treatments; however, because constitutive polyphenol oxidase was highest in low-N plants, the magnitude of induction was greatest in these plants (nearly 2.5-fold) (Figure 4).

Experiment 2. The second experiment was similar to the first, but more limited in scope: no intermediate-N treatment was used, and all chemicals were assayed from the third leaf only. Jasmonic acid, a plant hormone involved in plant responses to wounding (Reinbothe et al., 1994), was used instead of caterpillar feeding to induce plants (Thaler et al., 1996). This was done to ensure that any differences in induction between nutrient treatments in the first experiment were not caused by differences in the intensity or rate of feeding of larvae on plants of the different nutrient treatments.

TABLE 2. ANOVA FOR EXPERIMENT 1 WITH PLANTS GIVEN ONE OF THREE LEVELS OF NITROGEN

	Constitutive phytochemicals							
	Protein				Total phenolics			
Source of variation	*df*	MS	*F* ratio	*P*	*df*	MS	*F* ratio	*P*
Fertilization (f)	2	8.43	7.94	0.001	2	8.45×10^8	94.05	<0.001
Leaflet position (lp)	2	27.55	25.97	<0.001	2	8.42×10^7	9.37	<0.001
f × lp	4	1.37	1.30	0.29	4	7.79×10^6	0.87	0.49
Error	43	1.06			44	8.98×10^6		
	Inducible phytochemicals							
	Polyphenol oxidase				Proteinase inhibitors			
	df	MS	*F* ratio	*P*	*df*	MS	*F* ratio	*P*
Damage (d)	1	0.899	30.49	<0.001	1	9.388	353.35	<0.001
Fertilization (f)	2	0.194	6.59	0.003	2	0.062	2.35	0.11
d × f	2	0.056	1.89	0.16	2	0.017	0.63	0.54
Error	41	0.030			41	0.027		

[a] Phenolics and total protein were analyzed from undamaged plants at three positions on the plant (first, third, and fourth leaves from the cotyledons). Polyphenol oxidase and proteinase inhibitors were analyzed from third leaves of damaged and undamaged plants of all fertilization levels.

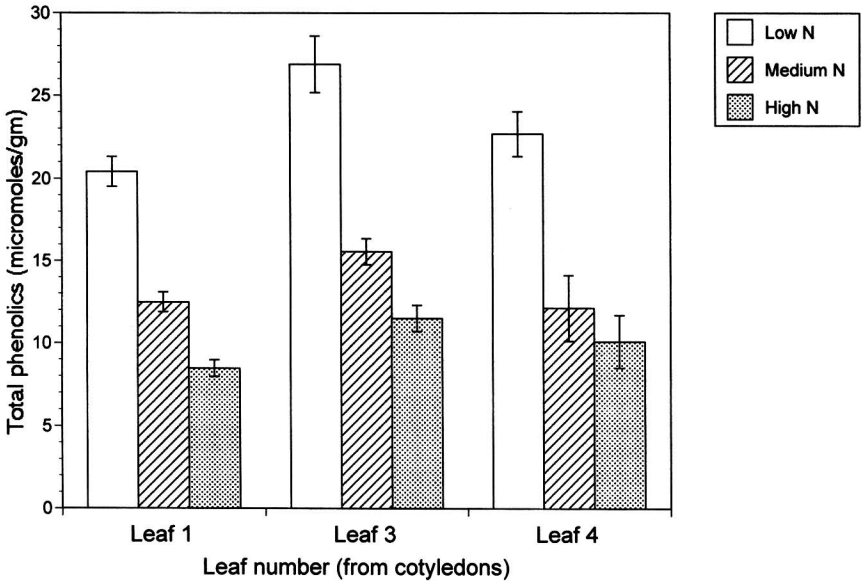

FIG. 2. Effect of nitrogen availability on concentrations of total phenolics μmoles/gm fresh weight ± 1 SE) at three leaf positions in four-leaf tomato plants. Fertilization regimes are described in the text. Leaf 4 was the youngest leaf; leaf 1 the oldest.

Protein levels were higher, and phenolic levels lower, in high-N plants than in low-N plants. Leaflets from low-N plants contained 2.5 ± 0.2% protein (wet weight); leaflets from high-N plants contained 5.3 ± 0.7% protein (wet weight) (difference statistically significant; $P = 0.004$). The corresponding figures for phenolics were 8898 ± 396 nmol/g gresh wt in low-N plants and 4316 ± 320 nmol/g in high-N plants (difference significant; $P < 0.001$).

As in the first experiment, fertilization regime had no effect on the inducibility of proteinase inhibitors (Figure 5, Table 3): induced plants of both fertilization regimes exhibited high proteinase inhibitor activities. Activities of proteinase inhibitors were slightly higher in low-N plants than in high-N plants, particularly in uninduced plants (Figure 5, Table 3, significant fertilization effect).

The patterns of polyphenol oxidase expression in sprayed and unsprayed plants were similar to the patterns found in the first experiment (Figure 6; Table 3). Polyphenol oxidase activities in control (unsprayed) plants were somewhat higher in low-N plants than in high-N plants (Table 3). Activities of polyphenol oxidase were not, however, statistically different in sprayed versus

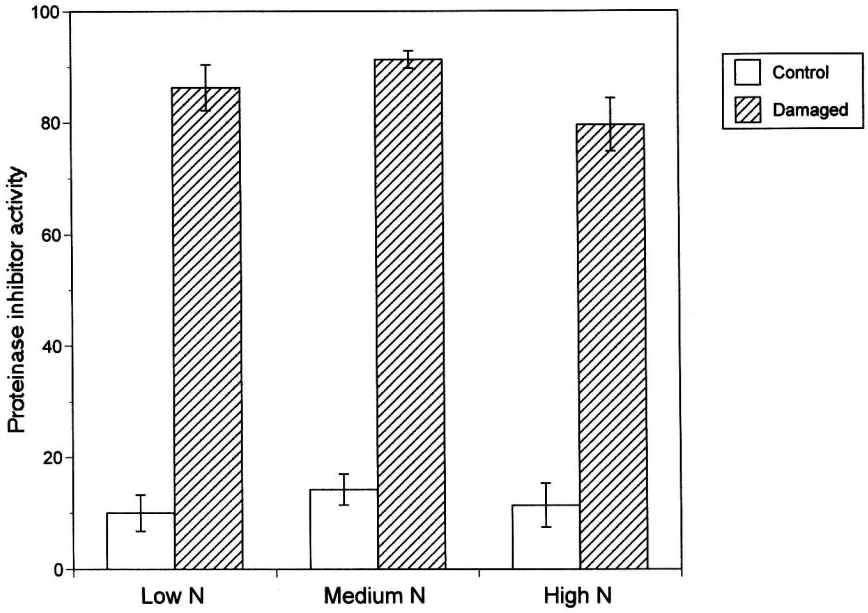

FIG. 3. Effect of nitrogen availability on activities of proteinase inhibitors (% inhibition of chymotrypsin ± 1 SE) in undamaged leaflets of damaged third leaves and from corresponding leaflets on undamaged plants.

unsprayed plants. Polyphenol oxidase activities in sprayed plants of the two fertilization treatments were nearly identical.

DISCUSSION

Allocation to secondary metabolism in plants is influenced by physiological constraints imposed by resource availability as well as by ecological demands for increased allocation to defense in certain tissues at certain times. Some theories of plant defense have emphasized the former (e.g., the C/N balance hypothesis), while other theories have emphasized the latter (e.g., various optimal defense theories). Recent efforts have been made to integrate ecological and physiological explanations of patterns of allocation (Herms and Mattson, 1992; Lerdau et al., 1994), but experiments that simultaneously examine the influences of both resource availability and ecological factors on allocation to secondary metabolism are rare. In the experiments reported here, we examined the separate and interactive effects of nitrogen availability and damage/chemical induction

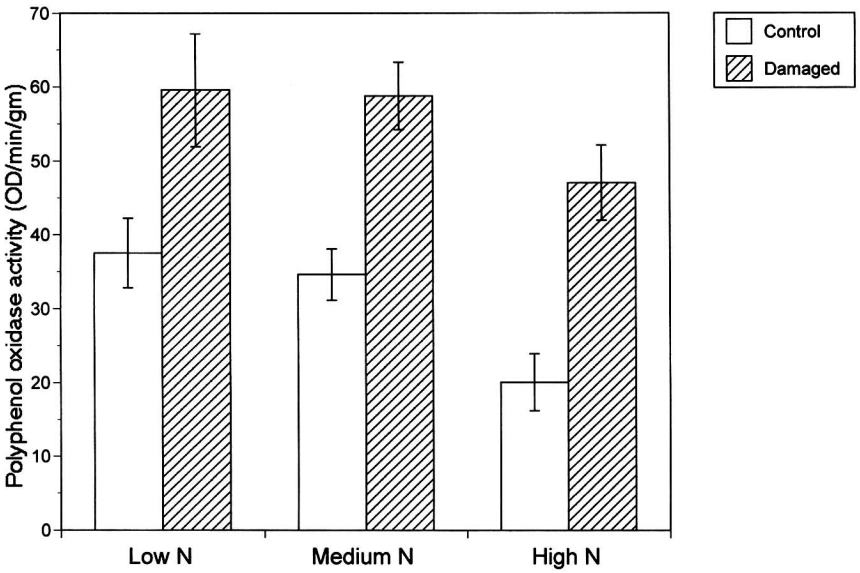

FIG. 4. Effect of nitrogen availability on activities of polyphenol oxidase (ΔOD/min/g wet weight ± 1 SE) in undamaged leaflets of damaged third leaves and from corresponding leaflets on undamaged plants.

on constitutive levels and inducibility of two inducible proteins, polyphenol oxidase and proteinase inhibitors, and on constitutive levels of phenolics, which are not inducible by damage in tomato (Stout, unpublished data; Stout et al., 1996a). Our results demonstrate that, while there clearly are physiological constraints on allelochemical expression in tomato foliage, ecological factors (in this case, demand for defense signaled by insect feeding) are possibly more important determinants of allocation to secondary metabolism.

The influence of nitrogen availability on secondary metabolism was most clearly demonstrated by levels of phenolics in undamaged plants subjected to various fertilization regimes. Plants given low levels of N had significantly higher levels of phenolics than plants given higher levels of N (Figure 2). Wilkens et al. (1996b) reported a similar response of phenolics in foliage of cherry tomato plants (*L. esculentum*) to different N regimes. Increases in phenolics under conditions of low N have been interpreted by some as a passive consequence of the increased flow of excess photosynthate into secondary metabolism (the C/N balance hypothesis) (Waterman and Mole, 1989; Reichardt et al., 1991). However, recent evidence indicates that the increase is actively controlled by the plant (Berenbaum, 1995); in tomato, for example, nutrient

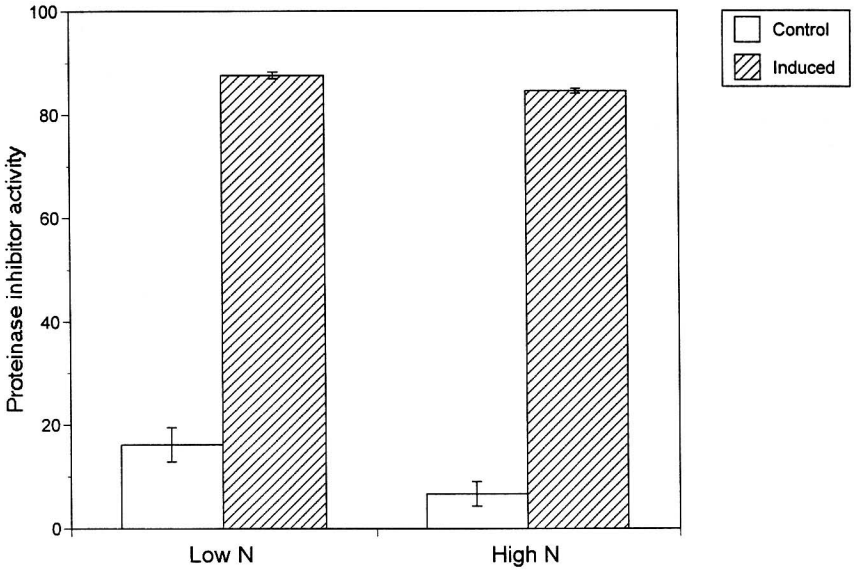

FIG. 5. Effect of nitrogen availability on activities of proteinase inhibitors (% inhibition of chymotrypsin ± 1 SE) in untreated plants and in plants treated with jasmonic acid to induce proteinase inhibitors.

stress increases the expression of several genes involved in phenolic metabolism (Bongue-Bartelsman and Phillips, 1995). An indication from our study that increases in phenolics in tomato foliage under N stress are controlled is the fact that, in both our study and that of Wilkens et al. (1996b), young leaves (which have higher concentrations of N and, hence, if allocation to secondary metabolism were strictly passive, would be expected to show lower allocation to secondary metabolism) had higher levels of phenolics than older leaves (Figure 2). This pattern is commonly observed (e.g., Frischknecht et al., 1986) and probably reflects an adaptive strategy aimed at protecting valuable but vulnerable young leaves (Herms and Mattson, 1992).

In contrast to the pattern observed for phenolics, the activities of proteinase inhibitors were, for the most part, unaffected by limitations in nitrogen availability. Activities of proteinase inhibitors in undamaged plants were low in both high-N and low-N plants—indeed, slightly higher in low-N plants than in high-N plants in the second experiment (Table 3, Figure 5). Furthermore, proteinase inhibitor activities in damaged plants were high regardless of fertilization regime. If allocation to secondary metabolism were contingent upon passive flow of carbon and nitrogen into secondary metabolism (Reichardt et al., 1991), then

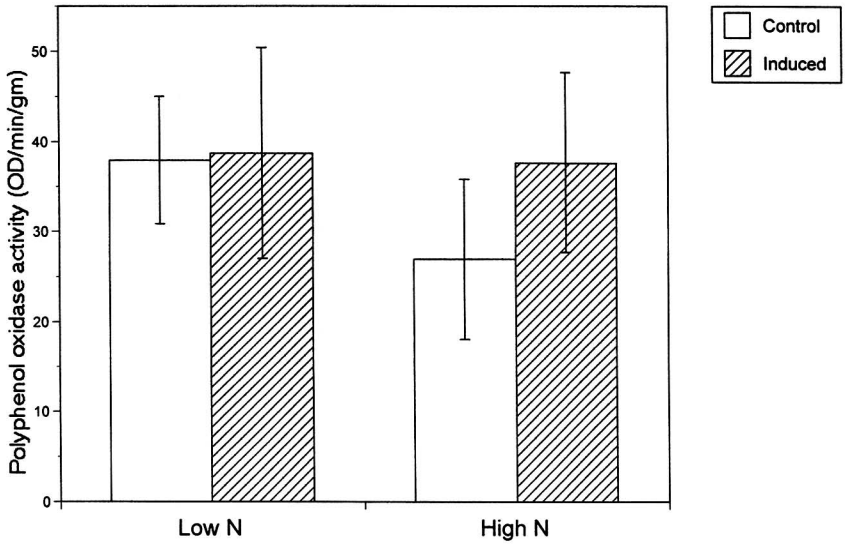

FIG. 6. Effect of nitrogen availability on activities of polyphenol oxidase (ΔOD/min/g wet weight ± 1 SE) in untreated plants and in plants treated with jasmonic acid to induce polyphenol oxidase. Data are not statistically different.

plants subjected to low levels of N would be expected to show an attenuation of chemical responses involving nitrogen-containing molecules. The insensitivity of proteinase inhibitor induction to fertilization regime is thus evidence for an active, damage-induced increase in allocation to defense that overrides physiological constraints. It must be noted, however, that effects of nitrogen availability on the rapidity and duration of the proteinase inhibitor response were not

TABLE 3. ANOVA FOR EXPERIMENT 2: EFFECTS OF FERTILIZATION (TWO LEVELS OF NITROGEN) ON INDUCIBILITY OF POLYPHENOL OXIDASE (PPO) AND PROTEINASE INHIBITORS (PI) BY JASMONIC ACID

	PPO				PIs			
Source of variation	*df*	MS	*F* ratio	*P*	*df*	MS	*F* ratio	*P*
Jasmonate (j)	1	0.048	3.31	0.08	1	5.655	373.27	<0.001
Fertilization (f)	1	0.058	4.03	0.05	2	0.077	5.11	0.03
j × f	1	0.050	3.50	0.07	2	0.026	1.72	0.20
Error	28	0.014			41	0.015		

investigated in our study; such effects have been found for alkaloid induction in lupine (Johnson et al., 1989).

As with proteinase inhibitors, patterns of polyphenol oxidase expression demonstrate an insensitivity to limitations in nitrogen availability. Absolute activities of polyphenol oxidase in leaflets from sand-grown plants were higher than in leaflets of similar age from soil-grown plants (Stout et al., 1996a,b), which almost certainly possess higher levels of foliar N than the low- and medium-N plants used in this study (Stout, unpublished data). In addition, constitutive polyphenol oxidase activities were higher in low-N plants than in high-N plants. Finally, inducibility of polyphenol oxidase was not completely abolished even in low-N plants (particularly evidenced by the results of the first experiment; Table 2). The smaller differences between constitutive and inducible polyphenol oxidase activities in low-N plants than in high-N plants (Figures 4 and 6) evidently did not result from an inability of plants to produce polyphenol oxidase—levels of polyphenol oxidase were quite high—but probably reflect a trade-off between constitutive polyphenol oxidase activities and inducibility of polyphenol oxidase. A negative correlation between constitutive levels and inducibility of polyphenol oxidase has been previously reported (Stout et al., 1996b).

The effect of nitrogen availability on chemical induction has been investigated in at least three other plant species. Baldwin et al. (1993) found that alkaloids in 59-day-old tobacco plants subjected to low levels of N were still inducible by damage, but that alkaloids in plants deprived of N for 15 days before damage were not. Johnson et al. (1989) reported that the alkaloid response of *Lupinus suuculentus* to mechanical damage was slower and somewhat attenuated in nitrogen-stressed plants. Zangerl and Berenbaum (1995) found no effect of nitrogen availability on induction of furanocoumarins (which do not contain N) in wild parsnip (*Pastinaca sativa*). Thus, the limited data available suggest that chemical responses involving natural products that contain N are constrained, but not abolished, by limitations in N availability.

A number of theories have been put forward to account for patterns of allelochemical expression within and among plants. In tomato, as in many other plants (Berenbaum, 1995), none of these theories is, by itself, capable of fully explaining observed distributions of secondary chemicals. The GDBH, which has received much recent attention, predicts an increase in levels of secondary chemicals under conditions that, like moderate nutrient stress, limit plant growth to a greater extent than photosynthesis (Herms and Mattson, 1992; Lerdau et al., 1994). In our study, patterns of expression of constitutive polyphenol oxidase, phenolics, and (to a lesser extent) proteinase inhibitors conformed to this prediction of the GDBH. The utility of the GDBH in explaining constitutive patterns of expression of phenolic compounds has been previously noted in many plant species (Waterman and Mole, 1989; Herms and Mattson, 1992; Beren-

baum, 1995), but the applicability of the GDBH to enzymatic and proteinaceous defenses had not previously been tested. The GDBH, in contrast, makes no specific predictions regarding the effect of nitrogen availability on induced responses. The damage- or jasmonate-induced increases in proteinase inhibitors and damage-induced increases in polyphenol oxidase in our experiments are best understood as responses to the ecological demand for defense (Lerdau et al., 1994) signaled by feeding by *H. zea* or application of jasmonate. In tomato, this ecological demand seems to hold a certain primacy, such that the response is largely unaffected by nitrogen availability even though the response involves molecules that contain N.

Enzymatic and proteinaceous defenses such as polyphenol oxidase and proteinase inhibitors are important (Duffey and Felton, 1991; Duffey and Stout, 1996) but have only recently received appropriate attention. Enzymatic/proteinaceous defenses possess several characteristics that may allow them to more easily circumvent factors that constrain other types of secondary compounds. Unlike phenolics and many other secondary metabolites, proteinaceous defenses are primary gene products and do not require extensive machinery for their metabolism. In addition, enzymatic/proteinaceous defenses are often effective at lower concentrations than other types of secondary metabolites because their mechanisms of defensive action involve catalysis and/or initiation of self-propagating processes (e.g., redox, polymerization, peroxidation). Hence, the amount of nitrogen incorporated into these types of compounds may not be significant even in nitrogen-limited plants. Proteinase inhibitors, for example, constitute only ca. 1% of leaf protein (300 μg/g wet wt leaf tissue) (Farmer et al., 1992; Ryan et al., 1986) in damaged plants, and, in an analysis by Gershenzon (1994), proteinase inhibitors had markedly lower costs on a per gram basis than other types of secondary metabolites. Finally, enzymatic/proteinaceous defenses may also play a role in primary metabolism. In particular, polyphenol oxidase, which is located primarily in plastids, almost certainly plays a role, albeit currently unknown, in primary metabolism (Kuwabara et al., 1997); the elevated activities of polyphenol oxidase in nitrogen-stressed plants may be part of the tomato's physiological adaptation to low nitrogen.

Our results underscore the complementary nature of theories that emphasize physiological constraints on expression of defense and those that emphasize ecological influences; a combination of both types of explanations probably accounts for patterns of allocation to secondary metabolites in tomato. In general, the extent to which expression of a natural product is contingent upon primary metabolism (resource availability) probably depends on a number of factors, including rate of turnover of the compound, typical concentration of the compound in plant tissue, elemental composition of the compound, metabolic pathways involved in synthesis of the compound, and role(s) of the compound in primary metabolism or defense against pathogens and abiotic stresses. Impor-

tantly, our results show that enzymatic and proteinaceous defenses may be less subject to physiological constraints than the low-molecular-weight organic compounds that have traditionally occupied most of the attention of chemical ecologists.

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COMPARATIVE ELECTROPHYSIOLOGICAL STUDIES OF OLFACTION IN PREDACEOUS BUGS, *Podisus maculiventris* and *P. nigrispinus*

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Abstract—Electroantennograms (EAGs) were recorded from both sexes of spined soldier bug (SSB), *Podisus maculiventris*, and Brazilian SSB (BSSB), *P. nigrispinus* to determine antennal olfactory responsiveness of 23 compounds in SSB and 14 compounds in BSSB, including the multicomponent male-produced aggregation pheromone and plant volatiles. EAGs of both species were similar. (*E*)-2-Hexenol and (*E*)-2-hexenal elicited the greatest EAGs, followed by heptanol, nonanal, hexanal, and the pheromonal compounds, (±)-α-terpineol and benzyl alcohol. Both sexes of SSB and BSSB were more sensitive to components of the male-produced aggregation pheromone [(±)-α-terpineol, (±)-linalool, and benzyl alcohol] and nonanal than either (*E*)-2-hexenol or (*E*)-2-hexenal (a component of the aggregation pheromone). BSSB were more sensitive to (±)-α-terpineol, (±)-linalool, benzyl alcohol, and nonanal than were SSB. EAGs to the plant volatile 1-hexanol and the pheromonal components (*E*)-2-hexenal and (±)-α-terpineol decreased significantly with removal of antennal segments, suggesting that receptors for these compounds are distributed over the distal three segments of the five-segmented antennae.

Key Words—Pheromone, plant volatile, olfactory receptors, electroantennogram, predators, *Podisus maculiventris*, *Podisus nigrispinus*, insect.

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INTRODUCTION

Sexual orientation of true bugs to their mates was first investigated systematically by Mitchell and Mau (1971). These investigators showed that male southern green stink bugs, *Nezara viridula* (L.), release a pheromone that attracts conspecific males and females, as well as parasitoids. This pioneering study was extended by the identification of the specific chemicals responsible for the observed attraction (Aldrich et al., 1987).

The spined soldier bug, *Podisus maculiventris* (Say) (Heteroptera: Pentatomidae) (SSB), and Brazilian spined soldier bug, *P. nigrispinus* (Dallas) (BSSB), are common generalist predators (O'Neil, 1988; McPherson, 1982) in areas of North America (Aldrich et al., 1984a) and South America (Freitas et al., 1990), respectively. Predaceous bugs are important because inundative releases of species such as *P. maculiventris* and *Perillus bioculatus* (F.) may be used to manage a number of pestiferous insects including the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Hough-Goldstein and Keil, 1991; Hough-Goldstein and McPherson, 1996).

The dorsal abdominal glands (DAGs) associated with abdominal segments 3 and 4 of male *P. maculiventris* and *P. nigrispinus* release a blend of seven chemicals that attracts conspecifics and associated parasitoids (Aldrich et al., 1984a). These glands in both species have the same compounds, but in different proportions. The blend of chemicals is attractive not only to adults, but also to nymphs (Sant'Ana et al., 1997). Although many compounds of bugs have been identified (Gilby and Waterhouse, 1965; Games and Staddon, 1973; Ishiwatari, 1974, 1976; Aldrich, 1988, 1995; Blumenthal, 1978; Slaymaker and Tugwell, 1984), few studies have investigated peripheral reception of these compounds by antennal receptors (Chinta et al., 1994; Dickens et al., 1995).

We report here results of investigations to determine selectivity and sensitivity of antennal receptors of both sexes of SSB and BSSB to components of the male-produced aggregation phermone of SSB. As both species produce the same compounds but in different proportions in their attractant glands, comparative studies between the two were done to determine similarities in olfactory reception. Since the putative pheromone components are common plant odors, antennal olfactory responsiveness of both species was determined for related plant odors and their analogs. To localize pheromone responsive neurons, electroantennogram recordings were made following ablation of terminal antennal segments.

METHODS AND MATERIALS

Insects. Colonies of *P. maculiventris* and *P. nigrispinus* were started from insects captured in pheromone-baited traps at the USDA-ARS, Beltsville Agri-

cultural Research Center, Beltsville, Maryland, and Universidade de Visçosa, Brazil, respectively. All insects were reared in a growth room at 26 ± 2°C, 60% ± 10% relative humidity, and 16:8 L-D photoperiod, at the USDA-ARS, Insect Chemical Ecology Laboratory, Beltsville, Maryland. Insects were provided with water vials, green bean pods, and pupae of *Tenebrio molitor* (Coleoptera: Tenebrionidae).

Odorous Stimuli. Chemicals tested were chosen based on either their presence in the segment 3–4 dorsal abdominal gland (DAG) of SSB and BSSB males or their occurrence in plants of the prey's habitat (p-h) (see Table 1). Also tested were SSB dorsal abdominal gland extract (DAG) from adult males, a topical ether extract of *Heliothis virescens* larvae, and a synthetic pheromone blend composed of 53.6% (*E*)-2-hexenal + 40% (±)-α-terpineol + 4.9% benzyl alcohol + 0.9% (±)-linalool + 0.5% (+)-terpinen-4-ol for SSB, and 3.58% (*E*)-2-hexenal + 84% (±)-α-terpineol + 3.4% benzyl alcohol + 2.81% (±)-linalool ± 1.82% (+)-terpinen-4-ol + 4.4% *trans*-piperitol for BSSB. Purities of all compounds were determined using gas chromatography.

Stimulus dilutions in nanograde hexane were delivered from glass odor cartridges (80 mm long × 5 mm ID) as 5-μl aliquots on Whatman No. 1 filter paper (7 × 18 mm). Odor cartridges were oriented towards the antenna from a distance of 1 cm. Odor molecules evaporating from the filter paper were carried over the preparation by dry, charcoal-filtered, hydrocarbon-free air. Stimulus duration was 1 sec; interstimulus time intervals of 2–3 min allowed for recovery of the sensory cells. Air around the experimental set up was continuously exhausted.

Because of the wide range in volatilities of test compounds (see Kovats indices, Table 1) (Kovats, 1961), only relative comparisons can be made between the odorous stimuli except for closely related compounds, between which comparisons are valid.

Electrophysiology. Electroantennogram (EAG) techniques utilized in these studies are a modification of previous techniques (Schneider, 1957) and are described in detail elsewhere (Dickens, 1984; Dickens et al., 1995). Briefly, Ag–AgCl capillary electrodes filled with *Drosophila* ringer (NaCl 7.5 g/liter, KCl 0.35 g/liter, and $CaCl_2$ 0.21 g/liter) were used. Intact insects were immobilized on a cork block using adhesive tape. After puncturing the antenna with a tungsten needle in the proximal and distal segments, the recording electrode was inserted into the distal antennal segment, and the indifferent electrode was placed into the proximal segment. The signal was amplified by a Grass P-16 microelectrode DC preamplifier and viewed on a storage oscilloscope. EAGs were recorded on a stripchart recorder.

Experimental Protocol. Three types of experiments were performed. The first two elucidated selectivity and sensitivity of antennal receptors of *P. maculiventris* and *P. nigrispinus* for male-produced pheromone components, prey-

TABLE 1. CHEMICALS USED IN EAG EXPERIMENTS, THEIR PURITY, SOURCE OF SUPPLY, KOVATS' RETENTION INDICES, AND BIOLOGICAL SOURCE

Chemical	Purity (%)	Source of supply[a]	I_k[b]	Identified from insect (I), host plant (II)[c]
Green leaf volatiles (GLVs)				
(*E*)-2-Hexenal	>90	A	965	I 1/II 2, 3, 4, 5, 6, 7
Hexanal	>94	A	945	II 2, 7
I-hexanol	99	A	980	II 2, 7
(*E*)-2-Hexenol	>99	A	995	II 2, 3, 4, 5, 7
(*E*)-2-Hexenoic acid	100	A	na[d]	II 7
(*E*)-2-Hexenyl acetate	100	C	na	II 7
Monoterpenes				
Myrcene	>50	A	1115	II 3, 4, 5, 6
(±)-Linalool	>99	A	na	I 1/II 3, 4, 5, 6
Nerol	>98	B	1410	II 2, 5
Geraniol	>88	B	1425	II 2, 3, 4, 5
(±)-α-Terpineol	>97	A	1340	I 1/II 2
(+)-Terpinen-4-ol	>94	A	na	I 1
trans-piperitol	>60	D	na	I 1
Benzenoid compounds				
Benzyl alcohol	100	A	1260	I 1/II 2
Benzaldehyde	>89	A	1172	I 1/II 4
2-Methyl benzyl alcohol	>99	C	na	
Aldehydes				
Heptanal	>90	A	1040	II 3, 5, 7
Octanal	100	A	na	II 7
Nonanal	>96	A	1225	II 3, 5
Tetradecanal	>60	C	na	I 1 (nymphs)
Other				
n-Tridecane	>97	A	na	I 1 (nymphs)

[a] A. Aldrich Chemical Co., Milwaukee, Wisconsin; B. Pfalz & Bauer, Inc., Stamford, Connecticut; C. Fluka Chemical AG, Buchs, Switzerland; PCR Research Chemical Inc., Gainesville, Florida.
[b] Kovats indices [Kovats, (1961), Hedin et al. (1976)].
[c] 1, Aldrich et al. (1948b); 2, Hedin et al. (1971); 3, Hedin et al. (1973); 4, Hedin et al. (1975); 5, Hedin et al. (1976); 6, Loughrin et al. (1994); 7, MacLeod et al. (1982).
[d] Not available.

habitat volatiles, and a prey odor extract. The third localized receptors for one plant odor and two pheromone components to specific antennal segments.

In the first experiment, the general responsiveness of antennal receptors to individual odorants was measured by recording EAGs to volatiles emanating from 50-μg stimulus loads of each compound. Presentation of each odorant was randomly ordered for each sample. In both SSB and BSSB, six replicates were obtained for each sex.

In the second experiment, odorants were selected for more detailed examination based on data obtained in the first experiment. Dose–response curves were constructed from EAGs elicited by serial dilutions of each compound (0.005–500 μg). Serial dilutions were presented in order from the lowest to the highest stimulus load. Three replicates were obtained for each sex for both species.

A third experiment localized pheromone and plant odor receptors in SSB and BSSB to certain segments of their five-segmented antennae. EAGs were recorded from adult males and females in which the distal segment of the antenna were removed and from adult males and females in which both the distal and penultimate segments of the antenna were removed. EAGs obtained in this experiment were compared to EAGs obtained from intact antennae. The recording electrode was always placed at the end of the last intact antennal segment. This experiment was replicated three times for both sexes of SSB and BSSB. Odorous stimuli included 50-μg stimulus loads of 1-hexanol (a plant odor) and (*E*)-2-hexenal and α-terpineol (pheromone compounds). Different individuals were used for each EAG experiment.

1-Hexanol (50-μg stimulus load) was used as a standard to normalize all responses, so that responses within an individual and among individuals could be compared (Payne, 1975). Stimulation with the standard preceded and followed every two stimulations. Millivolt responses were converted into a percentage of the mean of two nearest responses to the standard. Control stimulations (5 μl of the hexane solvent) were made at the beginning and at the end of each preparation. The mean response to the control was subtracted from each EAG. Each EAG was measured as the peak of depolarization during the stimulation period.

The threshold was considered to be the lowest dose at which the lower limit of the standard error of the mean response is greater than the upper limit of the standard error for the lowest dilution tested. Saturation level was taken as the highest dose at which the mean response is equal to or less than the previous dose.

Statistical Analyses. EAGs were compared statistically using analysis of variance procedure and Duncan's multiple range test (Duncan, 1955). Sexual differences between points on dose–response curves were compared for significant differences in sex using the *t* test for two means (Ostle, 1969).

RESULTS

Responses to Standard. Mean electroantennograms (EAGs) to the standard, 1-hexanol (50-μg stimulus load) in *Podisus maculiventris* males (0.5 mV $\pm$ 0.014; $N = 22$), were significantly greater than those obtained for females (0.45 mV $\pm$ 0.015; $N = 22$). *P. nigrispinus* females (0.55 mV $\pm$ 0.015; $N = 14$)

were more responsive to 1-hexanol than males (0.46 mV ± 0.012; $N = 14$) ($P < 0.005$).

Antennal Chemoreceptive Selectivity. EAGs were significantly different ($P < 0.05$) for the individual compounds tested in the SSB and BSSB antennae. Differences between males and females also occurred in the magnitude of EAGs for individual odorants (Tables 2 and 3).

In *Podisus maculiventris* (SSB), the green leaf volatiles (GLVs), (*E*)-2-hexenol and (*E*)-2-hexenal, were, in general, the most effective odorants tested. Among monoterpenes, α-terpineol elicited the greatest EAGs in SSB adults (Table 2). Significant differences in EAGs between sexes in this class of compounds were observed to (±)-α-terpineol, geraniol, (+)-terpinen-4-ol, myrcene, and *trans*-piperitol. Females were more responsive to these monoterpenes than males, except males exhibited a higher response to myrcene. Among the benzenoids tested, benzyl alcohol was the most effective. Both benzyl alcohol and benzaldehyde elicited significantly higher responses in SSB females than males. Overall responses to 6-, 7-, and 9-carbon aldehydes were higher than those elicited by 8- and 14-carbon aldehydes. Females were generally more responsive to 6-, 7-, 8-, and 9-carbon chain compounds than were males. However, the differences proved to be significant only for heptanal and octanal. EAGs of both sexes increased to a maximum at 7-carbon chain length.

The synthetic pheromone blend of SSB elicited greater EAGs in both sexes than did the DAG extract, although no significant difference was found between sexes. *Heliothis virescens* larval extract was barely detected by the antennal receptors of SSB males or females.

In *Podisus nigrispinus* (BSSB), GLVs were the most effective compounds tested (Table 3). The greatest responses were elicited by (*E*)-2-hexenal and (*E*)-2-hexenol. However, no differences in EAGs between sexes were recorded for these two compounds. Responses to oxygenated monoterpenes [(±)-linalool, nerol, (+)-terpinen-4-ol, and *trans*-piperitol)] were not significantly different between sexes. However, EAGs to (+)-α-terpineol were higher in males than in females. Responses to benzaldehyde were similar in both sexes, but males were more selective to benzyl alcohol than females. Males exhibited significantly higher responses to hexanal and heptanal than females. EAGs to hexanal, heptanal, and nonanal obtained from females were not significantly different.

Mean response to the BSSB pheromone blend did not differ significantly between sexes. *n*-Tridecane, a compound present in the gland of nymphs, elicited small EAGs from antennal receptors of adults.

Interspecific Comparison of Antennal Selectivity. In general, no statistically significant differences in EAGs of the same sex were observed between species to most of the compounds tested with one exception: significantly greater EAGs were elicited in response to the monoterpenol, α-terpineol, and heptanal in female SSB compared with female BSSB.

TABLE 2. MEAN EAGS OF SIX MALE AND SIX FEMALE *Podisus maculiventris* TO 50-μg STIMULUS LOAD OF SELECTED PLANT VOLATILES, PHEROMONE COMPOUNDS, AND RELATED CHEMICALS[a]

Males			Females		
Chemical	Mean EAGs[b]	(±SE)	Chemical	Mean EAGs[b]	(±SE)
(*E*)-Hexenol	75.82 ± 15.14	a	(*E*)-2-Hexenol	68.25 ± 18.1	a
(*E*)-2-Hexenal	65.7 ± 9.41	ab	**Heptanal**	60.71 ± 10.31	ab
P. maculiventris (synthetic)	45.75 ± 5.52	bc	Nonanal	55.05 ± 14.52	abc
Heptanal[c]	43.30 ± 4.38	cd	(*E*)-2-Hexenal	54.47 ± 14.43	abc
Nonanal	41.89 ± 2.77	cde	*P. maculiventris* (synthetic)	52.72 ± 8.28	abc
Hexanal	41.26 ± 4.43	cdef	**(±)-α-Terpineol**	51.25 ± 9.50	abcd
(±)-α-Terpineol	33.74 ± 5.33	cdefg	**Benzyl alcohol**	46.82 ± 11.94	abcde
Benzyl alcohol	27.23 ± 2.91	cdefg	Hexanal	45.94 ± 7.14	abcde
(*E*)-2-Hexenyl acetate	25.74 ± 4.9	cdefgh	**Benzaldehyde**	37.69 ± 8.54	bcdef
(*E*)-2-Hexenoic acid	23.44 ± 2.64	cdefgh	**Octanal**	33.01 ± 7.89	cdefg
P. maculiventris (gland)	22.65 ± 4.92	cdefgh	**Geraniol**	30.00 ± 6.20	cdefg
(±)-Linalool	21.38 ± 3.49	cdefgh	**(±)-Terpinen-4-ol**	27.91 ± 5.08	cdefgh
Benzaldehyde	18.82 ± 1.85	defgh	**trans-Piperitol**	25.35 ± 5.60	defgh
Geraniol	18.67 ± 4.23	defgh	(*E*)-2-Hexenyl acetate	22.13 ± 5.28	efgh
Terpinen-4-ol	18.48 ± 3.66	efgh	(*E*)-2-Hexenoic acid	20.96 ± 3.88	efgh
Tetradecanal	16.9 ± 3.68	fgh	*P. maculiventris* (gland)	19.84 ± 4.36	efgh
Nerol	15.05 ± 2.39	gh	(±)-Linalool	16.76 ± 3.64	fgh
Myrcene	14.94 ± 0.96	gh	Tetradecanal	14.81 ± 2.24	fgh
2-Methyl benzyl alcohol	13.71 ± 3.1	gh	Nerol	13.72 ± 2.38	fgh
Octanal	12.81 ± 3.39	gh	2-Methyl benzyl alcohol	11.93 ± 1.92	fgh
***trans*-Piperitol**	10.33 ± 2.16	gh	**Myrcene**	8.15 ± 3.02	gh
Heliothis virescens odor	0.93 ±	h	*Heliothis virescens* odor	0.25 ± 0.24	h

[a]Numbers followed by different letters are significantly different within sex ($P < 0.05$; Duncan's multiple-range test).
[b]EAGs are represented as percent response to the standard = 1-hexanol (50 μg).
[c]Response to chemicals in bold type differ significantly between sexes.

TABLE 3. MEAN EAGS OF SIX MALE AND SIX FEMALE *Podisus nigrispinus* TO 50-μg STIMULUS LOAD OF SELECTED VOLATILES, PHEROMONE COMPOUNDS, AND RELATED CHEMICALS[a]

Males			Females		
Chemical	Mean EAGs[b]	(±SE)	Chemicals	Mean EAGs[b]	(±SE)
(*E*)-2-Hexenal	90.06 ± 20.34	a	(*E*)-2-Hexenal	82.81 ± 14.03	a
(*E*)-2-Hexenol	85.58 ± 14.38	a	(*E*)-2-Hexenol	80.61 ± 13.64	a
Heptanal[c]	56.56 ± 12.78	b	Nonanal	41.96 ± 5.45	b
Hexanal	51.55 ± 10.85	b	(+)-Terpinen-4-ol	38.68 ± 8.74	b
(±)-α-Terpineol	49.01 ± 4.89	bc	**Heptanal**	36.50 ± 4.11	b
P. nigrispinus (synthetic)	44.90 ± 10.87	bcd	*P. nigrispinus* (synthetic)	34.89 ± 3.61	b
Nonanal	40.41 ± 5.08	bcde	**Benzyl alcohol**	26.94 ± 3.79	b
Benzyl alcohol	38.81 ± 6.98	bcde	**Hexanal**	25.27 ± 4	b
(+)-Terpinen-4-ol	27.74 ± 6.52	cde	**(±)-α-Terpineol**	24.6 ± 3	b
(±)-Linalool	26.92 ± 6.86	cde	Benzaldehyde	24.58 ± 5.45	b
Benzaldehyde	26.15 ± 6.72	cde	(±)-Linalool	19.73 ± 3.56	b
Nerol	22.05 ± 3.93	de	Nerol	19.24 ± 2.45	b
n-tridecane	22.87 ± 4.27	e	*n*-tridecane	17.94 ± 2.96	b
trans-Piperitol	19.81 ± 3.93	e	*trans*-Piperitol	17.45 ± 2.74	b

[a]Numbers followed by different letters are significantly different within sex ($P < 0.05$; Duncan's multiple-range test).
[b]EAGs are represented as percent response to the standard = 1-hexanol (50 μg).
[c]Response to chemicals in bold type differ significantly between sexes.

Antennal Chemoreceptive Sensitivity. Podisus maculiventris dose–response curves with (*E*)-2-hexenal (Figure 1A) and (*E*)-2-hexenol (Figure 1B) showed receptors for both sexes to be equally sensitive to these compounds (threshold = 50 μg). Responses of males to the 50-μg dose were significantly greater than responses of females. Both sexes were less sensitive to (*E*)-2-hexenal or (*E*)-2-hexenol than to the remaining compounds, which reached threshold at the ca. 5-μg dose. EAGs of males to (±)-linalool were significantly greater than those of females (Figure 1F).

In *Podisus nigrispinus* threshold responses for (*E*)-2-hexenal (Figure 2A) and (*E*)-2-hexenol (Figure 2B) were similar for those obtained for SSB. However, threshold responses of BSSB to the other compounds occurred between the 0.5-μg and 5-μg stimulus loads (Figure 2C–F).

Interspecific Comparison of Antennal Sensitivity. Overall, slopes of dose–response curves for the selected odorants in SSB were similar to those in BSSB. Usually, responses increased from the lowest to the highest dose presented to

the antennae. Dose–response curves for (*E*)-2-hexenal (Figure 2A) and (*E*)-2-hexenol (Figure 2B) were nearly identical for both species. Receptors for nonanal, (±)-α-terpineol, benzyl alcohol, and (±)-linalool in SSB were less sensitive than those in BSSB (Figures 1C–F, 2C–F).

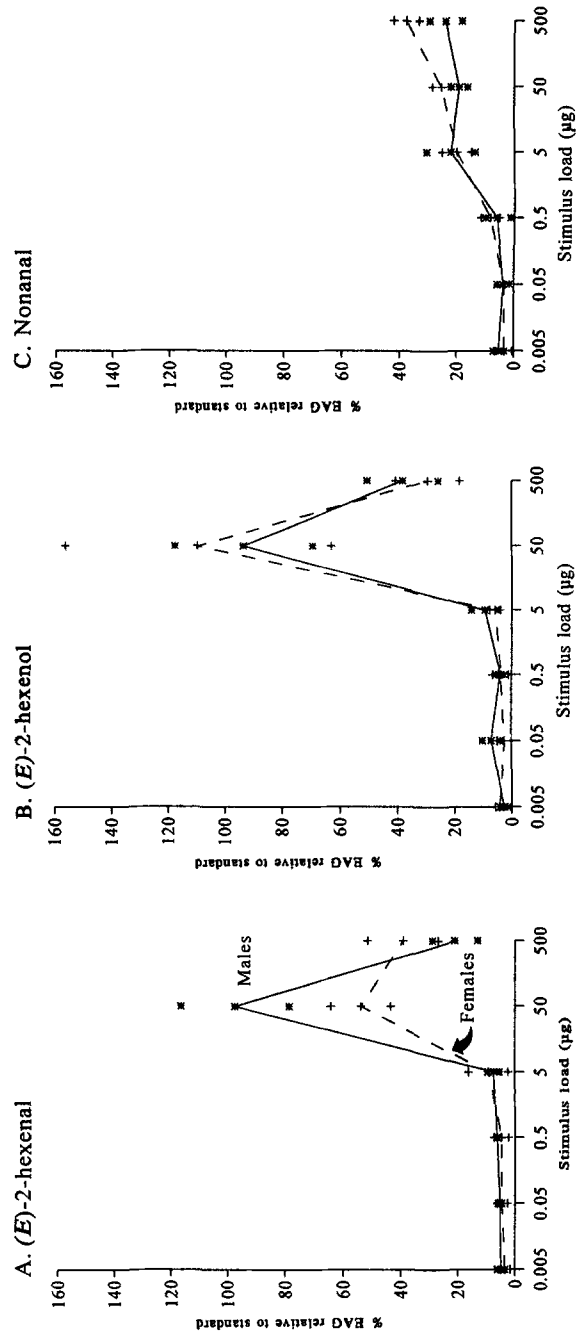
Localization of Antennal Receptors. In general, extirpation of one or two antennal segments resulted in decreased EAGs to the plant odor, 1-hexanol, and two pheromone components, (+)-α-terpineol (*E*)-2-hexenal; with one exception, responses of BSSB males to (+)-α-terpineol with the distal segment removed did not differ from responses from intact antennae (Figure 3). EAGs recorded from the three proximal segments were smaller than those recorded from the whole antenna and the antenna with distal segment extirpated.

DISCUSSION

Antennal Chemoreceptive Selectivity. Generally, (*E*)-2-hexenal and (*E*)-2-hexenol elicited the greatest EAGs for both SSB and BSSB. Hexan-1-ol, (*E*)-2-hexenol, (*Z*)-3-hexenol, and their derivatives, e.g., (*E*)-2-hexenal (also a pheromone component), are components of green odor and are referred to as green leaf volatiles (GLVs) (Visser et al., 1979). Since the release of GLVs increases after insect feeding (Whitman and Eller, 1990; Dicke et al., 1990), the presence of these volatiles in the air could be used by predators as a cue to find prey that are feeding on plants.

Responses of *P. maculiventris* and *P. nigrispinus* to (*E*)-2-hexenol and (*E*)-2-hexenal could be based in two aspects. First, the compounds have relatively low molecular weights and high volatilities based on Kovats relation indices (Table 1). However, this may not be the main factor for such high responses since hexanal is more volatile and EAGs elicited by it were smaller. Second, (*E*)-2-hexenal is a component of the pheromonal attractant for both SSB and BSSB, and GLVs, in general, may be attractants both to other phytophagous insects (Visser and Ave, 1978) and may enhance activity of insect pheromones (Dickens, 1989; Dickens et al., 1990, 1993; Light et al., 1993). Thus, specific sensitivity to these odorants would facilitate location of prey by *Podisus* through deciphering attractant semiochemical messages from them as shown for an insect parasitoid (Whitman, 1988; Whitman and Eller, 1990).

The lack of response of antennal receptors in predaceous bugs to larval washes of lepidopterous larvae, such as *H. virescens* in our current study, might indicate the use of plant volatiles for location of prey habitat by these generalist predators. Once in the area of potential prey, the bug may use other senses to pinpoint its prey. For example, substrate vibrations produced by prey feeding may serve as directional cues for *P. maculiventris* in search of prey (Pfannenstiel et al., 1995). Another method is used by the predaceous clerid beetle, *Thana-*


A. (E)-2-hexenal
B. (E)-2-hexenol
C. Nonanal
% EAG relative to standard
Stimulus load (µg)
Males
Females
0
20
40
60
80
100
120
140
160
0.005
0.05
0.5
5
50
500

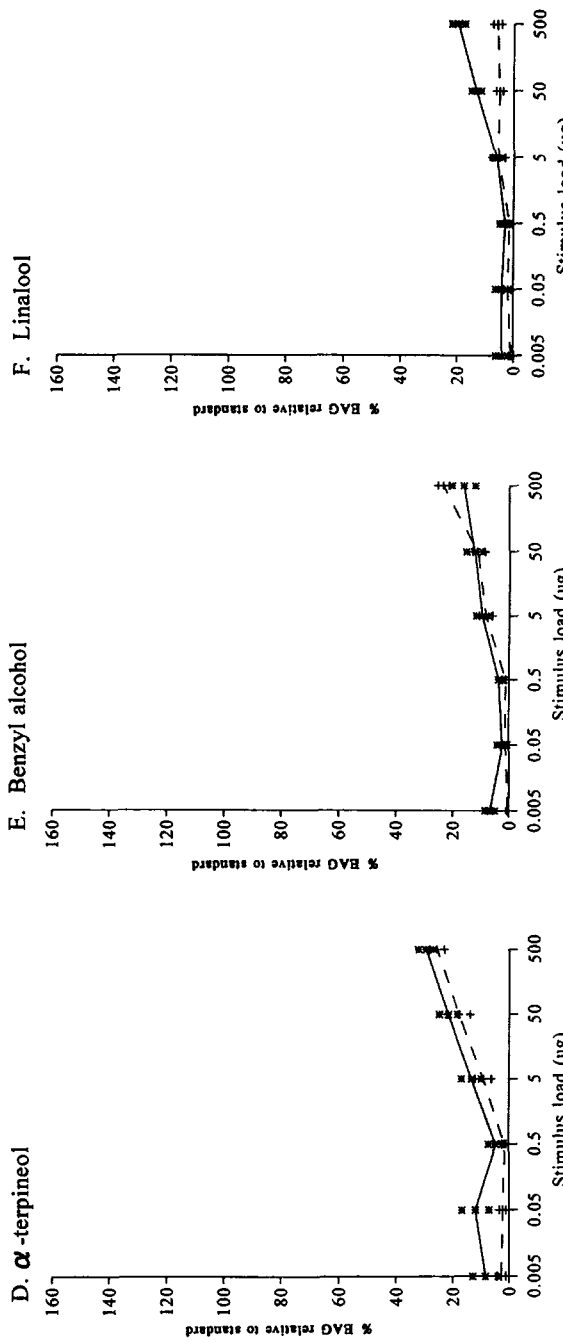
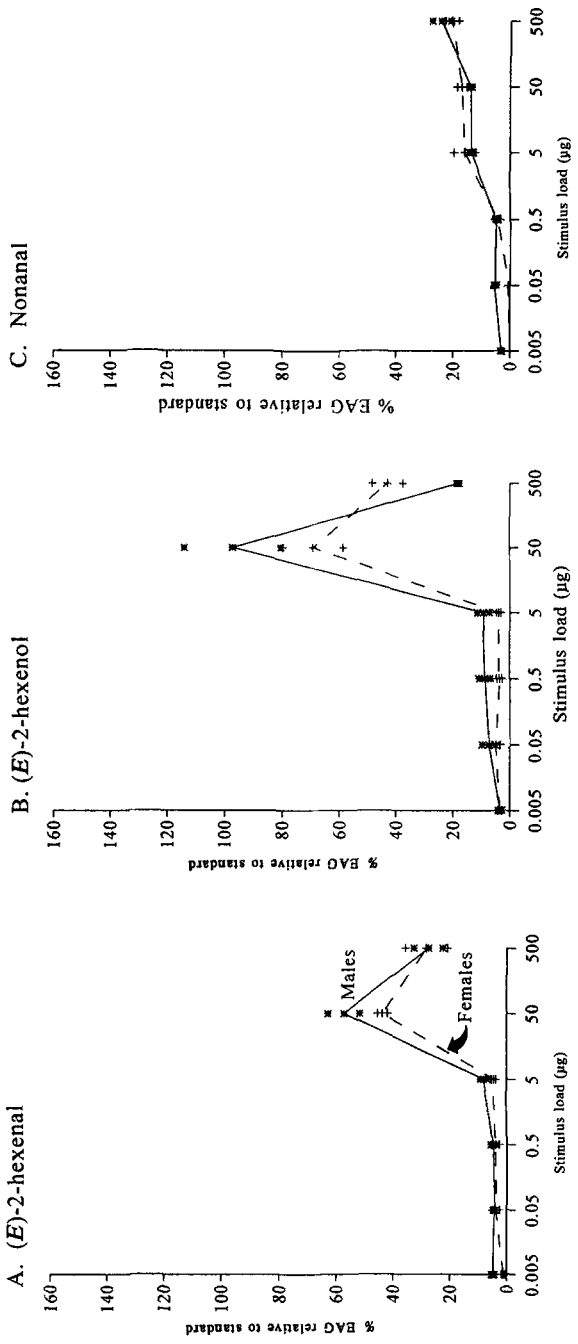


FIG. 1. Dose–response curves constructed from mean EAGs of male and female *Podisus maculiventris* to ascending serial stimulus loads of selected insect and plant odorants: (A) (*E*)-2-hexenal, (B) (*E*)-2-hexenol, (C) nonanal, (D) (±)-α-terpineol, (E) benzyl alcohol, (F) (±)-linalool. Markers above and below points on dose–response curves represent ± SE ($N = 3$; * males, $^+$ females).


A. (E)-2-hexenal
B. (E)-2-hexenol
C. Nonanal
% EAG relative to standard
Stimulus load (µg)
0.005
0.05
0.5
5
50
500
0
20
40
60
80
100
120
140
160
Males
Females

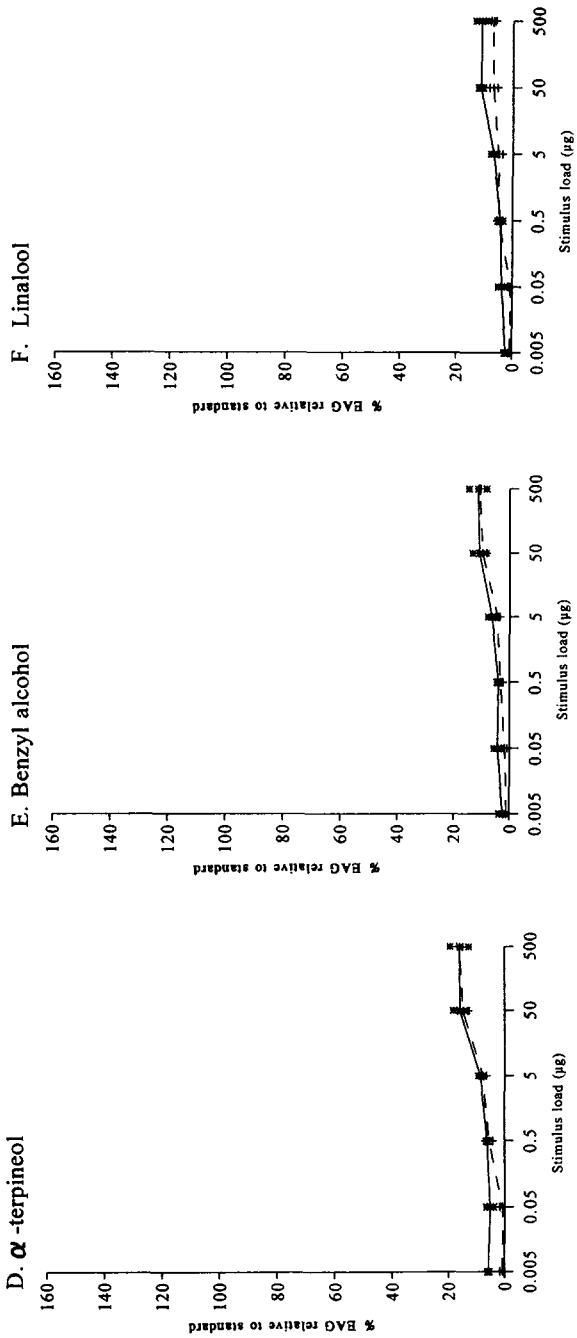
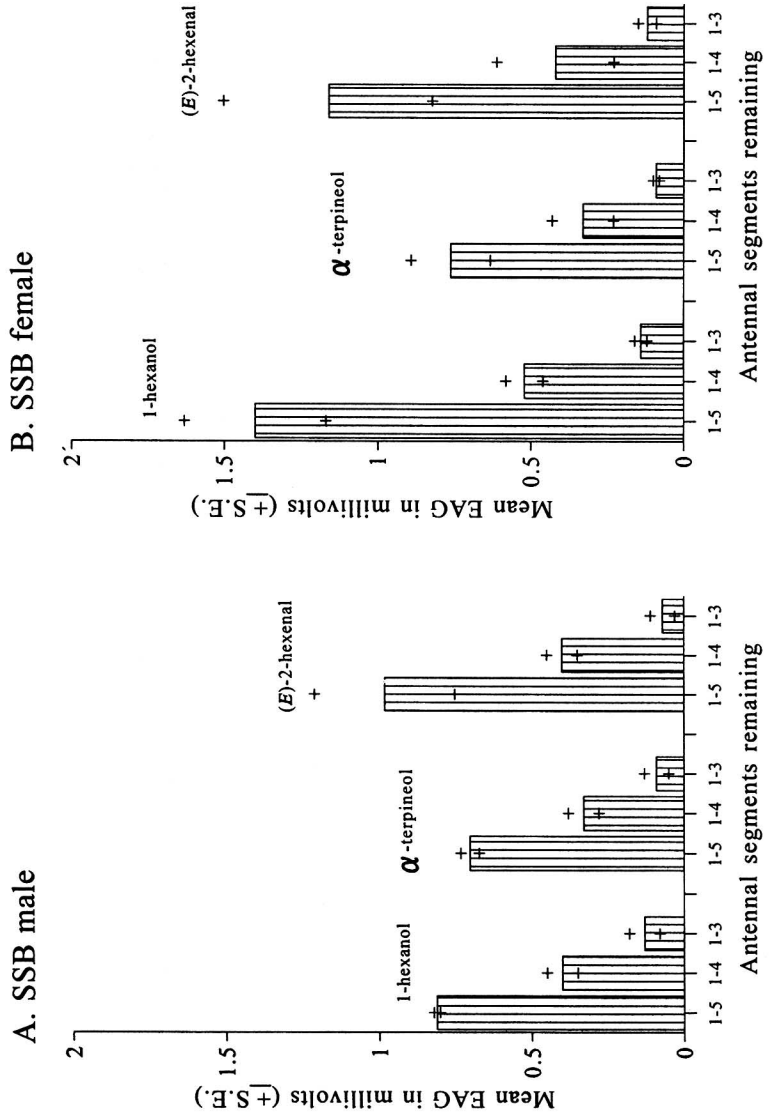


FIG. 2. Dose–response curves constructed from mean EAGs of male and female *Podisus nigrispinus* to serial stimulus loads of selected insect and plant odorants: (A) (*E*)-2-hexenal, (B) (*E*)-2-hexenol, (C) nonanal, (D) (±)-α-terpineol, (E) benzyl alcohol, (F) (±)-linalool. Markers above and below points on dose–response curves represent ± SE ($N = 3$; * males; $^{+}$ females).


A. SSB male
B. SSB female
Mean EAG in millivolts (± S.E.)
2
1.5
1
0.5
0
1-hexanol
α-terpineol
(E)-2-hexenal
1-5 1-4 1-3
Antennal segments remaining

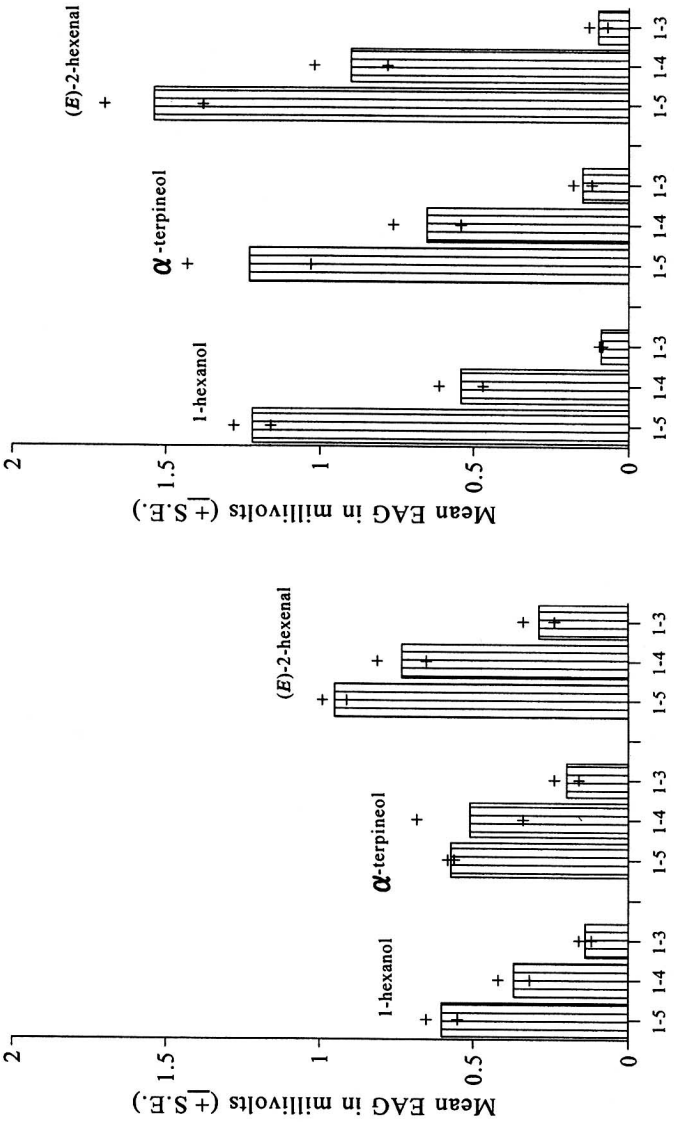


FIG. 3. Mean EAGs in millivolts (± SE, $N = 3$) elicited from *Podisus maculiventris* males (A), females (B), and *P. nigrispinus* males (C), females (D) with whole antennae, segments 1–4 only, and segments 1–3 only in response to a plant odor (1-hexanol), and two pheromone components [(±)-α-terpineol and (*E*)-2-hexenal] at 50-μg stimulus load. Markers above and below bars represent ± SE ($N = 3$).

simus dubious, which has receptors tuned to the aggregation pheromone of its prey, the southern pine beetle, *Dendroctonus frontalis* (Payne et al., 1984).

The second most active class of compounds in our study, the aldehydes heptanal, hexanal, and nonanal, are known plant volatiles (Hedin et al., 1976; Light et al., 1988). These compounds could be used by predators, such as SSB and BSSB, and parasites as cues for prey habitat location. Antennal receptors of some parasitoids respond to these aldehydes and their alcohol derivatives (*Microplitis demolitor*, Ramachandran and Norris, 1991; *Campoletis sonorensis*, Baehrecke et al., 1989; and *Microplitis croceipes*, Li et al., 1992). Alternatively, the high sensitivity to plant odors could also be explained by the fact that many of these compounds are the same as or closely related to pheromones, and, thus, may stimulate some pheromone receptors.

Antennae were more responsive to (±)-α-terpineol and benzyl alcohol than to related monoterpenes and benzenoid compounds (Table 1). Significant responses to these compounds reflect their importance as pheromone components for both SSB and BSSB (Aldrich et al., 1984a, 1991).

Sexually dimorphic responses to compounds such as (±)-α-terpineol may be explained by differences in the number and distribution of functional sensillar and/or receptor neuron types. While sexual differences in the number and distribution of sensilla do occur (Dickens and Sant'Ana, unpublished), receptor neurons associated with them have not been characterized. Variations in responses to the same compound by SSB and BSSB may be related to functional adaptations with regard to the relative importance of certain odorants in chemical communication schemes for SSB and BSSB. Similarities in EAGs of SSB and BSSB to plant odors and most pheromonal compounds may be explained by similar acceptor populations in both species.

Antennal Chemoreceptive Sensitivity. Both SSB and BSSB showed significant sexual differences in sensitivities to plant volatiles and pheromonal compounds. Dose–response curves for (*E*)-2-hexenal and (*E*)-2-hexenol for SSB did not differ from those observed for BSSB, thus indicating similar detection mechanisms for these compounds. Thresholds for each compound were the same for both species (50-μg stimulus load). EAGs peaked at the 50-μg stimulus load before decreasing significantly at the 500-μg stimulus load. Such a decrease in response may be due to decreased volatility at the higher dose or to unexplained physiological phenomena.

SSB and BSSB males and females were, in general, more sensitive to (±)-α-terpineol, benzyl alcohol, (±)-linalool, and nonanal (Figures 1 and 2), which are part of the pheromone blend released by males to attract females and other males (Aldrich et al., 1984a). Nonanal is present in small amounts in the dorsal abdominal gland of female SSB, but a behavioral role has not been assigned to it (Aldrich et al., 1984b). Sensitivity of both sexes of SSB and BSSB to nonanal observed here indicate its potential role as a chemical messenger.

Differences in relative saturation levels and sensitivities in dose–response curves in SSB and BSSB for (*E*)-2-hexenol and (*E*)-2-hexanal (a pheromone component) (Figures 1A and B, 2A and B) compared to the pheromone components, (±)-α-terpineol, benzyl alcohol, and (±)-linalool are striking (Figures 1D and E, 2D and E). These differences may be due to differences in relative volatilities [(±)-α-terpineol, (±)-linalool and benzaldehyde are less volatile than (*E*)-2-hexenol and (*E*)-2-hexanal] or in numbers of receptive sensilla. Since pheromone components are of critical importance to species propagation, a few sensilla, sensitive and specifically tuned to them, would effectively enhance detection of these important signals in an inherently noisy environment.

Localization of Receptors for Plant Volatiles and Pheromones. Directly proportional relationships between EAG amplitudes and the length of antenna exposed to the stimulus (or in this case number of antennal segments remaining) indicates that receptors for each of these odorants are distributed over each of the terminal two segments (Figure 3). Similar results have been found for several other insects including: *Argyrotaenia velutinana* (Roelofs and Comeau, 1971), *Trichoplusia ni* (Mayer et al., 1984), *Oryzaephilus surinamensis* (White, 1991), and *Acyrthosiphon pisum* (Giessen et al., 1994).

Although EAGs continued to decrease after removing the two distal segments, small, but significant, EAGs could still be recorded for each of the chemicals tested indicating that receptors for these odorants are present on segments 1–3 (Figure 3). In the tarnished plant bug, *Lygus lineolaris* (Miridae), EAGs to a plant odor (1-hexanol) and an insect-produced odor [(*E*)-2-hexenyl butyrate] did not decrease after removal of the distal antennal segment (Dickens et al., 1995). This suggested that receptors for both odorants tested were housed principally on the second and third segments of the four-segmented antenna, as was shown in behavioral bioassays of sexual attraction in a closely related species, *L. hesperus* (Graham, 1988).

In conclusion, receptor selectivity and sensitivity of SSB and BSSB to environmental volatiles and pheromone compounds are similar. GLVs and other plant volatiles are used by SSB and BSSB together with the male-produced aggregation pheromone (which has components that are also present in plant emissions) as cues for mating, location of hosts, feeding, and oviposition. Chemical attractants for predators would be useful in programs of integrated pest management (IPM) using natural enemies (Sant'Ana et al., 1997). Such selective use of semiochemicals to assemble and keep SSB in potato fields could lessen damage by the Colorado potato beetle (Hough-Goldstein and McPherson, 1996).

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NOVEL CHITINOLYTIC ENZYMES WITH BIOLOGICAL ACTIVITY AGAINST HERBIVOROUS INSECTS

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Abstract—The soil bacteria, *Streptomyces albidoflavus*, secretes endochitinases and chitobiosidases that are active over a broad range of pH (4–10). Ingestion of this mixture of chitinolytic enzymes significantly reduced the growth and development of *Trichoplusia ni* and significantly reduced survival of *Myzus persicae*, *Bemisia argentifolii*, and *Hypothenemus hampei*. Perfusion chromatography was used to separate endochitinases from chitobiosidases. The endochitinases had significantly greater biological activity against *Bemisia argentifolii* than the chitobiosidases. The utility of chitinolytic enzymes as regulators of populations of herbivorous insects is discussed.

Key Words—Endochitinase, chitobiosidase, *Trichoplusia ni*, *Bemisia argentifolii*, *Hypothenemus hampei*, *Myzus persicae*.

INTRODUCTION

A major research effort has focused on chitinolytic enzymes as a phytochemical defense in plants against plant pathogenic fungi (Boller, 1985; Broglie et al., 1991). This research indicates that: (1) chitinolytic enzymes from plants are potent inhibitors of fungal spore germination and mycelial growth in vitro (Broekaert et al., 1988; Mauch et al., 1988; Roberts and Selitrennikoff, 1988;

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Schlumbaum et al., 1986), (2) resistant cultivars produce higher levels of chitinolytic activity than susceptible cultivars (Hughes and Dickerson, 1991; Vogelsang and Barz, 1990), and (3) plants that have been transgenically enhanced for chitinolytic activity are more resistant to selected pathogenic fungi than nontransformed plants (Broglie, et al., 1991). In addition, virulence of a pathogen appears to be related to the rate of induction of plant chitinolytic enzyme(s) (i.e., an avirulent pathogen induces plant chitinolytic enzymes more rapidly than a virulent pathogen) (Hedrick et al., 1988; Joosten and De Wit, 1989; Vogeli-Lange et al., 1988; Voisey and Slusarenko, 1989). All these findings support the hypothesis that chitinolytic enzymes significantly contribute to plant resistance against fungal pathogens.

While chitinolytic enzymes in plants appear to function as an effective phytochemical defense against plant pathogens, there is no evidence that plant chitinolytic enzymes are effective defensive agents against herbivorous insects. One explanation for this lack of biological activity is the pH requirements of these enzymes. Plant chitinolytic enzymes, in general, require an acidic environment for activity; they have little or no activity under alkaline conditions (Pegg and Young, 1982; Zhe-fu et al., 1992). However, herbivorous insects, in general, have alkaline midguts (Berenbaum, 1980; Grayson, 1951; Mishra and Sen-Sarma, 1987). Therefore, chitinolytic enzymes from plants probably are not active within the lumen of the insect's midgut, which will limit their biological activity when ingested by herbivorous insects that have alkaline digestive tracts. A search for chitinolytic enzymes that function in an alkaline environment resulted in the isolation and characterization of chitinolytic enzymes from *Streptomyces albidoflavus* (Broadway et al., 1995). This strain of bacteria secretes three classes of chitinolytic enzymes: (1) *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) (Bielka et al., 1984), which cleaves monomeric units from the terminal end of chitin, (2) 1,4-β-chitobiosidase, which cleaves dimeric units from the terminal end of chitin, and (3) endochitinase (EC 3.2.1.14) (Bielka, et al., 1984), which randomly cleaves the chitin molecule internally (Sahai and Manocha, 1993). Optimal activity for all three types of enzymes from *S. albidoflavus* occurred at pH 4–6; however, 55–74% of the chitobiosidase and endochitinase activity was detectable at pH 8–10. Chitobiosidase activity originates from two proteins that are strongly acidic (pI < 3.0) with molecular mass of 27 kDa and 34 kDa, while the endochitinase activity originates from five major acidic proteins (pI 5.1, 5.3, 5.7, 5.8–5.9, and 6.4) with molecular masses of 59, 45, 38.5, 27, and 25.5 kDa, respectively. The current work focuses on the biological activity of the chitobiosidases and endochitinases from *S. albidoflavus* against a range of herbivorous insects, including Lepidoptera [*Trichoplusia ni* (Hubner)], Coleoptera [*Hypothenemus hampei* (Ferrari)], and Homoptera [*Bemisia argentifolii* Bellows & Perring, and *Myzus persicae* (Sulzer)].

METHODS AND MATERIALS

Partial Purification of Alkaline Chitinolytic Enzymes. The strain of *Streptomyces albidoflavus* used for production of chitinolytic enzymes is accessioned in the ARS Culture Collection (Peoria, Illinois) as NRRL B-16746 (Broadway et al., 1995). Chitinolytic enzymes were produced by *Streptomyces albidoflavus* when grown in liquid medium containing 0.012% magnesium sulfate, 0.1% glucose, 0.1% calcium chloride, 0.05% manganese sulfate, 0.025% ferrous sulfate, 0.00125% zinc sulfate, and 0.5% crab shell chitin in 50 mM Tris, pH 9.0. Cultures were grown in flasks with constant shaking (250 rpm) at 30°C for four to five days. The biomass was removed from the broth by centrifugation at 6000*g* for 30 min at 4°C. The supernatant was filtered through mira cloth, then adjusted to 95% saturation with ammonium sulfate to isolate total protein. The mixture was incubated at 4°C, overnight, then centrifuged at 6000*g* for 30 min at 4°C. The pellet was resuspended in dH_2O and dialyzed against ice-cold dH_2O (130 × vol) to remove salt. The dialysate was centrifuged at 6000*g* for 10 min at 4°C to remove insoluble particles, and the supernatant was lyophilized. This powder was identified as semipurified chitinolytic enzyme mixture.

The endochitinases were separated from the chitobiosidases by perfusion chromatography (BioCAD Sprint, PerSeptive Biosystems, Cambridge, Massachusetts) on an HQ/M strong anion exchange column (4.6 × 100 mm), equilibrated with 20 mM Tris/bis-Tris propane, pH 9.0. A 5-mg sample of chitinolytic enzyme mixture was applied to the column, then endochitinases were eluted as two protein peaks: peak I was eluted with the Tris buffer and peak II was eluted with 80 mM NaCl in Tris buffer. The chitobiosidases were eluted with 300 mM NaCl in Tris buffer. The column was cleaned with buffer containing 2 M NaCl. Samples were collected in 2-ml fractions; fractions were pooled to combine protein(s) from a single peak. Each peak of protein was dialyzed against ice-cold dH_2O, lyophilized, then analyzed for total protein and enzyme activity. Then the proteins were applied to a nondenaturing PhastGel (procedure described below) to confirm the presence of endochitinase(s) and/or chitobiosidase(s). Based on these in vitro analyses, peaks I and II contained endochitinase activity, peaks IV and V had chitobiosidase activity, and peak III had endochitinase and chitobiosidase activity. (Note: Peak III was not used for bioassays against insects, because it contained both types of enzyme activity.)

Electrophoretic Analyses of Chitinolytic Enzymes. The PhastSystem electrophoresis unit (Pharmacia, Uppsala, Sweden) was used to characterize the proteins with chitinolytic activity. The number of proteins with chitinolytic activity was determined on a nondenaturing, discontinuous polyacrylamide gel (7.5% stacking gel, 20% separating gel, separation length 32 mm), using nondenaturing buffer strips containing 0.88 M L-alanine, 0.25 M Tris, pH 8.8. A 3-μl aliquot of the sample was mixed with 1 μl 4× sample buffer [0.25 M Tris,

pH 8.8, 0.008% bromophenyl blue (w/v)] and then transferred to a sample applicator for electrophoresis (following manufacturer's directions). Protein bands were visualized by staining the polyacrylamide gel with Coomassie blue (0.1% Coomassie R350 in 30% MeOH, 10% acetic acid). Proteins with chitobiosidase and endochitinase activity were detected on gels with an overlay containing 0.025% 4-methylumbelliferyl β-D-*N,N'*-diacetylchitobioside in 0.05 M Tris pH 9, 1% low melting DNA-grade agarose. The agarose-based mixture was boiled for 5 min, then cooled to 35°C. Immediately following electrophoresis, the agarose mixture was poured over the gel, and site(s) of enzyme activity appeared as fluorescent bands when exposed to UV light.

In Vitro Analyses of Enzymatic Activity and Total Protein. Endochitinase activity was measured by mixing a 500-μl aliquot of sample with 500 μl of 0.1 M acetate buffer, pH 5, containing 4% colloidal chitin. The mixture was shaken at 30°C for 24 hr. Then 5 ml of dH_2O was added to each tube, vortexed, and the optical density was measured at 510 nm. Percent reduction of turbidity was calculated for each tube. For calculation of specific activity, one unit was defined as the amount of enzyme required to obtain 1% reduction of turbidity under the above conditions.

Chitobiosidase activity was measured by mixing a 30-μl aliquot of sample with 50 μl of 0.1 M acetate buffer pH 5 containing 0.03% *p*-nitrophenyl β-D-*N,N'*-diacetylchitobiose in a well of a microtiter plate. The plate was incubated at 50°C for 15 min, then 50 μl of 0.4 M sodium carbonate was added to each well, and the optical density was measured at 410 nm. One nanokatal (nkatl) corresponds to the release of 1 nmol nitrophenyl per second under the above conditions. Data are reported as percentage nanokatals (i.e., nanokatals per 100 ml).

Prior to protein analyses, each fraction was dialyzed against dH_2O for 30 hr to remove salts. Total protein was determined for each fraction with Coomassie blue stain reagent (Pierce Chemical Co., Rockford, Illinois). Chitinase from *Serratia marcescens* (Sigma Chemical Co., St. Louis, Missouri) was used as a standard.

Insects. Eggs of *Trichoplusia ni* were provided by Dr. W. L. Roelofs (NY State Agricultural Experiment Station, Geneva, New York). To determine the effect of chitinolytic enzymes on larval growth and development, larval *T. ni* were reared on a high wheat germ-based meridic diet (Webb and Shelton, 1988) supplemented with chitinolytic enzymes. Each bioassay included four treatments (0, 0.25%, 0.5%, and 1% chitinolytic enzyme mixture), three cups per treatment, 20 neonate larvae per cup, and each bioassay was replicated three times. All larvae were weighed when controls reached the ultimate instar, and then monitored daily for developmental changes. The percent pupation and percent adult emergence was based on the total number of larvae weighed and total number of pupae recovered from each test diet, respectively.

Eggs of *Hypothenemus hampei* were provided by Dr. Alex Bustillo (National Center of Coffee Research, Chinchina, Colombia). Insects were maintained individually in wells of an ELISA plate, each well containing 0.2 ml of artificial diet (Villacorta and Barrera, 1993). Incubation conditions were maintained at 26°C, 60–70% relative humidity. Each bioassay included four treatments (0%, 0.25%, 0.5%, and 1% chitinolytic enzyme mixture), 20 eggs per treatment, and each bioassay was replicated three times. The insects were monitored for 30 days for developmental changes (from egg to adult) and mortality.

Myzus persicae were provided by Dr. Maurice Tauber (Department of Entomology, Cornell University, Ithaca, New York). Aphids were reared on turnip plants prior to use in experiments. Each bioassay consisted of six treatments (0%, 0.06%, 0.125%, 0.25%, 0.5%, and 1% chitinolytic enzyme mixture prepared in 20% sucrose), replicated four times each. Immature wingless aphids (~25) of similar age, were each placed in a glass cylinder arena (22 mm inner diameter, 21 mm height) covered on one end with parafilm. The other end of the arena was sealed when placed upright on a supporting substrate. An aliquot of 20% sucrose solution was added to the top of the parafilm, and another piece of parafilm stretched over the solution to form a thin layer of solution. The parafilm provided a membrane through which aphids could feed. Aphids were held for 24 hr in the cylinders to become acclimated to the test arena. After 24 hr the number of surviving aphids was recorded, the parafilm was replaced, and the sucrose solution was replaced with experimental chitinase solutions. Percent mortality was recorded at 24 and 48 hr after the chitinase solutions were added.

Whiteflies originated from a commercial greenhouse (Long Island, New York) on poinsettia in 1989 and were maintained on poinsettia in a colony at Cornell University. Adults of similar age were obtained by placing poinsettia leaves infested with pupae from the colony into sleeve cages provisioned with a poinsettia plant. Adults were allowed to emerge from pupae for one day before the remaining pupae were removed to prevent further adult emergence. Adults were then left in the cage on the poinsettia plant for an additional two days before use. Bioassays were conducted in polycarbonate vials (45 mm diameter, 74 mm high) over which parafilm was thinly stretched. A 0.5-ml aliquot of 10% sucrose solution, with or without chitinolytic enzymes, was placed onto the parafilm and covered by another tightly stretched layer of parafilm as with aphid trials. Twenty adult whiteflies were released into each vial through a small hole in the side of the vial and periodically evaluated for mortality over three days. Bioassays included a control treatment (10% sucrose) and either semipurified chitinase at 0.06, 0.125, 0.25, 0.5, and 1.0%, or one of five chitinase fractions at 0.5%. Each treatment was replicated three to six times for each bioassay. Aphid and whitefly trials were conducted on a laboratory bench under conditions of ~12L:12D and ~22°C.

Statistics. Analysis of variance (ANOVA) was used to test for significant effects of dietary chitinase treatments on insect mortality and development (Abacus Concepts, Inc., 1989). Percentage mortality data were transformed (arcsin of the square root of the proportion) prior to ANOVA. Where trials were repeated on different dates, date was considered a main factor along with treatment. Separation of treatment means was accomplished with the use of the least significance difference (LSD) test.

RESULTS

Purification of Chitinolytic Enzymes. The mixture of chitinolytic enzymes contained two proteins with chitobiosidase activity, and five proteins with endochitinase activity (Broadway et al., 1995). Strong anion exchange perfusion chromatography separated the endochitinases from the chitobiosidases, as shown by the elution profile of the column (Figure 1). Polyacrylamide gel electropho-

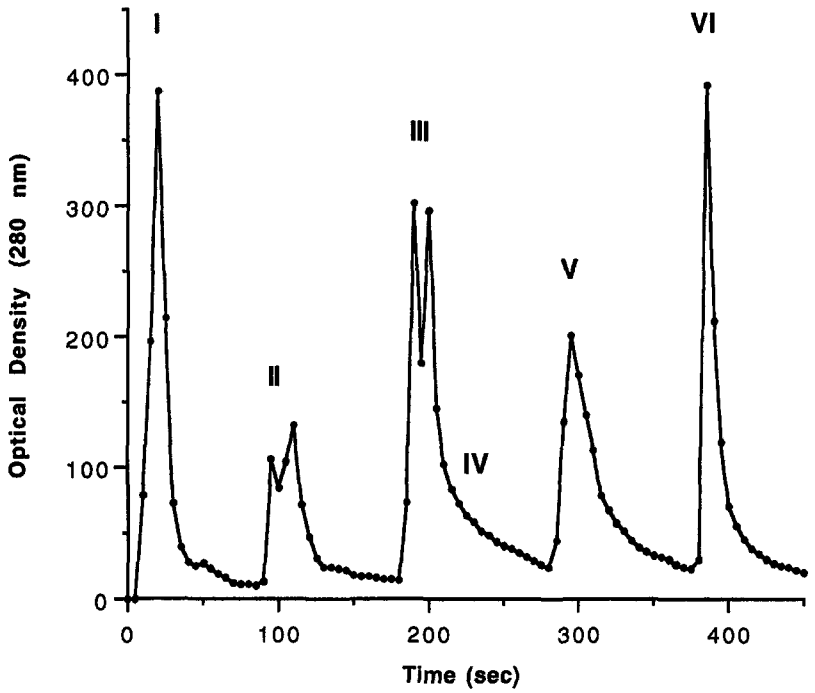


FIG. 1. Strong anion exchange perfusion chromatography of semipurified chitinolytic enzymes. Total chitinolytic activity, as measured by hydrolysis of *p*-nitrophenyl β-D-*N*,*N*′-diacetylchitobiose, was 0.14 nkat/peak I, 1.52 nkat/peak II, 0.26 nkat/peak III, <0.01 nkat/peak IV, 2.45 nkat/peak V, 0.09 nkat/peak VI.

resis enabled visualization of the number of bands of proteins in each peak (Figure 2a), and demonstrated that peaks I, II, and III had endochitinase activity, peaks III, IV, and V had chitobiosidase activity, while peak VI had no chitinolytic activity (Figure 2b).

Biological Activity of Alkaline Chitinolytic Enzymes. The lyophilized, chi-

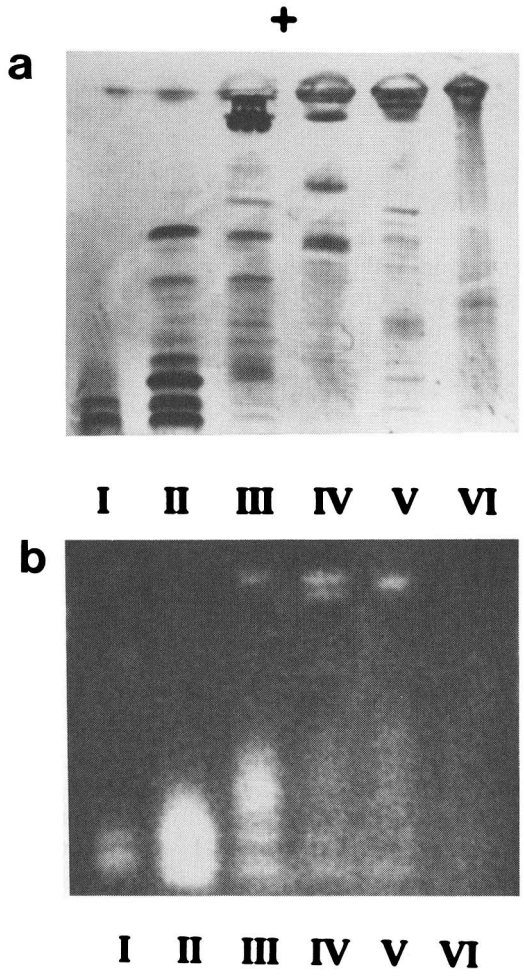


FIG. 2. Polyacrylamide gel electrophoresis of the peaks collected from anion exchange perfusion chromatography (Figure 1): (a) Coomassie stain to detect all proteins; (b) fluorogenic overlay to locate enzymes with chitinolytic activity. Based on previous experiments (Broadway et al., 1995), the upper (most acidic) two bands were chitobiosidases, while the lower (most alkaline) five bands were endochitinases.

tinolytic enzyme mixture contained 0.8–1.1 nkat of chitobiosidase activity/mg, and 154–165 units of endochitinase activity/mg. Dietary supplementation with the mixture of chitinolytic enzymes resulted in significant reductions in weight of larval *Trichoplusia ni* (expressed as percentage of the control) ($F = 121.56$, $df = 3,23$, $P < 0.001$). However, results of each of the three bioassays are presented (Figure 3a) rather than pooled data, because there was a significant interaction between date (bioassay number) and percent dietary intake of chitinase ($P = 0.016$). Results of each bioassay showed a similar inverse relationship between larval weight and dietary chitinase, although absolute values were variable at 0.25% dietary chitinase. Dietary supplementation with chitinases also resulted in significant reductions in percent pupation ($F = 34.69$, $df = 3,20$, $P < 0.001$), and percent adult emergence ($F = 6.64$, $df = 3,8$, $P = 0.015$) for *Trichoplusia ni* (Figure 3b and c). Due to the low number of individuals surviving to adulthood, ANOVA was conducted on data pooled across the three test dates.

In addition, ingestion of the mixture of chitinolytic enzymes resulted in a dose-dependent increase in mortality for *Myzus persicae* at 24 and 48 hr ($F = 20.33$ and 20.02, respectively; $df = 5,15$, $P < 0.001$), *Bemisia argentifolii* at 18 and 42 hr ($F = 11.23$ and 25.12, respectively; $df = 5,11$, $P < 0.001$) and *Hypothenemus hampei* ($F = 5.84$, $df = 3,8$, $P = 0.021$) at 30 days (Figure 4).

Separation of endochitinases from chitobiosidases resulted in significantly different levels of mortality for adult *B. argentifolii* ($F = 17.77$, $df = 5,19$, $P < 0.001$). Fractions containing endochitinase (peaks I and II) resulted in 80% and 50% mortality, respectively, after 36 hr of exposure, the fraction containing both proteins with chitobiosidase activity (peak IV) significantly elevated mortality to 38%, whereas mortality following exposure to a single (most acidic) chitobiosidase (peak V) did not differ from the untreated controls (peak VI and C) (Figure 5).

DISCUSSION

The control of insect pests on commercially grown crops relies predominantly upon the use of synthetic insecticides. However, in recent years, the use of these insecticides is increasingly regulated and limited because of their potential hazards to human health and the environment. As a result, there is an active search for alternative approaches to insect control that are target-specific and environmentally benign. One approach that is gaining significant attention is the use of agricultural cultivars that are resistant to pests. These cultivars can be developed by the transgenic introduction of target-specific natural resistance factors. However, to enhance host-plant resistance, we must first identify and characterize target-specific factors that will significantly reduce the population(s) of herbivorous insect(s).

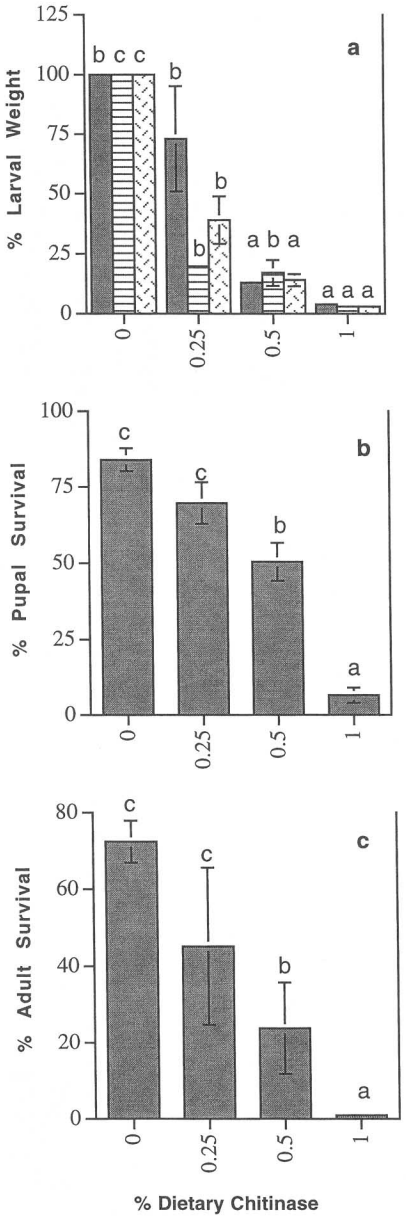
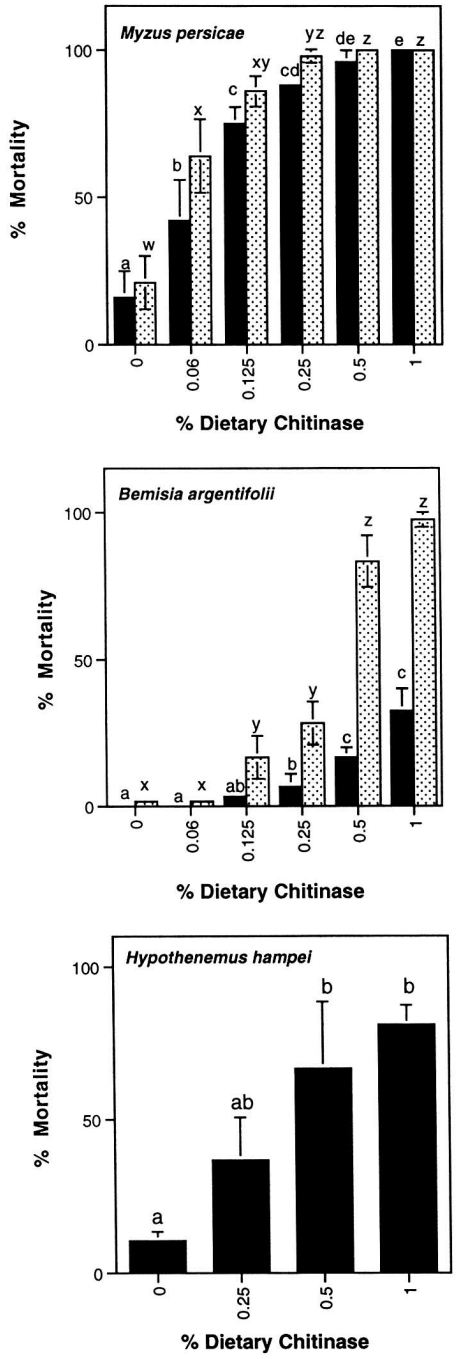


FIG. 3. Development of *Trichoplusia ni* on artificial diet supplemented with semipurified chitinolytic enzymes from *Streptomyces albidoflavus*. (a) Larval weight as a percent of the mean weight of untreated controls. Solid bars indicate weight of larvae in bioassay 1, striped bars are larvae in bioassay 2, hatched bars are larvae in bioassay 3. (b) Percent larvae that pupated. (c) Percent pupae that molted to adults. Vertical lines indicate ± 1 SEM. Columns associated with an insect growth stage having similar letters are not significantly different (LSD test).


Myzus persicae
% Mortality
100
50
0
a
w
b
x
c
xy
cd
yz
de
z
e
z
0
0.06
0.125
0.25
0.5
1
% Dietary Chitinase
Bemisia argentifolii
% Mortality
100
50
0
a
x
a
x
ab
y
b
y
c
z
c
z
0
0.06
0.125
0.25
0.5
1
% Dietary Chitinase
Hypothenemus hampei
% Mortality
100
50
0
a
ab
b
b
0
0.25
0.5
1
% Dietary Chitinase

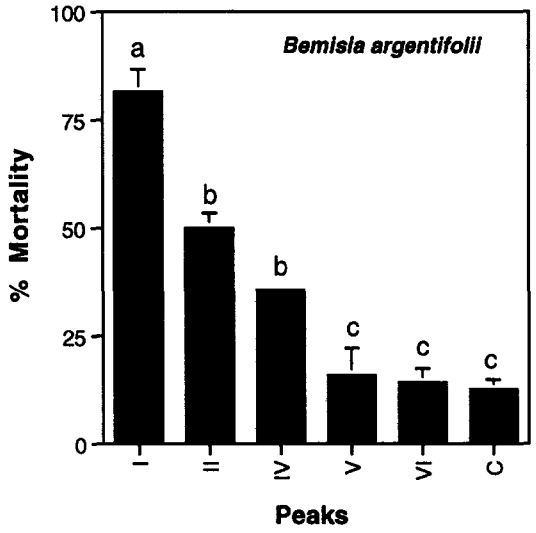


FIG. 5. The effect of dietary endochitinases or chitobiosidases on survival of *Bemisia argentifolii*. Endochitinase treatments I and II contained 10% sucrose and 0.5% peak I or II, respectively, from the anion exchange perfusion chromatography (Figure 1), while chitobiosidase treatments IV and V contained 10% sucrose and 0.5% peak IV or V, respectively, from the anion exchange column (Figure 1). Two controls were included: treatment VI contained 10% sucrose and 0.5% peak VI (no chitinolytic activity), and treatment C, which contained 10% sucrose. Vertical lines indicate ± 1 SEM. Columns with similar letters are not significantly different (LSE test).

Only a limited number of natural products have been characterized and identified as effective defensive agents against herbivorous insects. Few of these are proteins (e.g., proteinase inhibitors, arcelin, α-amylase inhibitors, lectins, endotoxin from *Bacillus thuringiensis*, and lipoxygenases), and even fewer are target-specific (Duffey and Felton, 1989; Gill et al., 1992; Hedin, 1983; Rosenthal and Janzen, 1979). Identification and characterization of proteins as resistance factor(s) enables the isolation of gene(s) that encode(s) these proteins.

FIG. 4. The effect of dietary chitinolytic enzymes on survival of *Myzus persicae*, *Bemisia argentifolii*, and *Hypothenemus hampei*. For *B. argentifolii*, solid bars indicate percent mortality after 18-hr exposure, stippled bars indicate percent mortality after 42-hr exposure. For *M. persicae*, solid bars indicate percent mortality after 24-hr exposure, while stippled bars indicate percent mortality after 48-hr exposure. For *H. hampei*, solid bars indicate percent mortality after 30-day exposure. Vertical lines indicate ± 1 SEM. Columns associated with a single time of observation and having similar letters are not significantly different (LSD test).

These genes can be transgenically inserted into agricultural crops, which may enhance the resistance of these crops against herbivorous insects without altering desirable characteristics of the cultivar(s) (Fraley et al., 1988; Hilder et al., 1987; Ryan, 1989; Vaeck et al., 1987).

A target-specific factor would reduce the risk to nontarget organisms (e.g., vertebrates). One target site that distinguishes arthropods from higher organisms is chitin. Chitin is a principal component of the insect's exoskeleton (and of the lining of the foregut and hindgut) and peritrophic membrane (which lines the midgut of many species of insects) and is essential for structural integrity of insects. Degredation of chitin located in the alimentary canal (i.e., foregut, hindgut, and/or peritrophic membrane) may significantly disrupt digestion and nutrient acquisition, in addition to other protective and/or physiological functions. For example, in some insects, the peritrophic membrane is thought to function as protection against abrasion of the gut wall (the site of absorption and digestion), in the compartmentalization of digestion in the midgut lumen, and as a barrier to pathogens and phytotoxins. Destruction of the peritrophic membrane may have a negative impact on all three of these functions that are essential for the survival of such insects.

The current study demonstrated that ingestion of an artificial diet containing a mixture of endochitinases and chitobiosidases will significantly reduce the growth and development of larval *Trichoplusia ni* and significantly decrease the survival of *Myzus persicae*, *Bemisia argentifolii*, and *Hypothenemus hampei*. Separation of the endochitinases from the chitobiosidases was accomplished by perfusion chromatography. Feeding studies indicated that the endochitinases dramatically reduced survival of *Bemisia argentifolii* (which may have been due to acute toxicity or repellency following ingestion), while the chitobiosidases had less of an effect on survival. These results are in contrast to previous work that demonstrated that the chitobiosidases from *Streptomyces albidoflavus* significantly reduced the growth and survival of the plant pathogenic fungi *Botrytis cinerea* and *Fusarium oxysporum* (Broadway et al., 1995).

The results of this study provided fundamental information required for evaluating the impact of chitinolytic enzymes on the growth and/or develoment of herbivorous insects. We now must demonstrate that chitinolytic enzymes, in planta, will reduce the growth and/or development of herbivorous insects. This information, followed by determination of the site of action and the physiological/physical effect of these ingested chitinolytic enzymes in vivo, will allow us to predict the long-term value of chitinolytic enzymes as defensive agents in plants.

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CHEMICAL AND BEHAVIORAL EVIDENCE FOR A THIRD PHEROMONE COMPONENT IN A NORTH AMERICAN POPULATION OF THE BLACK CUTWORM MOTH, *Agrotis ipsilon*

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Abstract—The sex pheromone of *Agrotis ipsilon* had been previously identified as a blend of (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) and (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac). A synthetic blend of *Z*7-12:Ac and *Z*9-14:Ac (30 μg:10 μg) is effective in attracting males in the field. In several countries, addition of (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) to the previously identified blend increases male captures, but this had not been demonstrated in North America. We found *Z*11-16:Ac, in addition to *Z*7-12:Ac and *Z*9-14:Ac, from pheromone gland extracts of females from North America. The mean ratio of *Z*7-12:Ac, *Z*9-14:Ac, and *Z*11-16:Ac produced by individual females was 70.5:14.2:15.3, respectively. In Kentucky, addition of *Z*11-16:Ac (60 μg) to a two-component blend of *Z*7-12:Ac and *Z*9-14:Ac significantly increased the trap capture rate in the field. Traps baited with this three-component blend were 3.3 times (1995) and 4.6 times (1996) more effective in capturing male *A. ipsilon* than the two-component blend. This improved effectiveness resulted in detection of *A. ipsilon* in 60% more of the sampling periods in the two years. In the wind tunnel, males flew upwind and contacted a rubber septum loaded with a three-component blend including *Z*11-16:Ac significantly more frequently than they did to any two-component blend. These results demonstrate that *Z*11-16:Ac is a pheromone component in this North American population of *A. ipsilon.*

Key Words—Lepidoptera, *Feltia jaculifera*, *Agrotis ipsilon*, sex pheromone, wind tunnel, pheromone traps, male attraction.

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INTRODUCTION

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), is a cosmopolitan moth that causes economic losses in many crops around the world (Rings et al., 1974; Clement et al., 1982, Radcliffe et al., 1991; Metcalf and Metcalf, 1993). In North America, *A. ipsilon* moths cannot overwinter in the northern states or Canada, where they occur during the summer months (Story and Keaster, 1982; Showers, 1997). Instead they migrate northward in the spring (Showers et al., 1989) and their progeny return to their southern overwintering range in the fall (Showers et al., 1993; Showers, 1997). The initial influx of *A. ipsilon* into northern areas is likely to be variable in time and magnitude. As a result, pheromone traps have become important to detect early occurrence of *A. ipsilon* in the northern range during their spring migration (Clement et al., 1981, 1982; Willson et al., 1981; Levine et al., 1982; Troester et al., 1983). In addition, pheromone traps could be useful to monitor population changes if captures of adult males are indicative of subsequent densities of larvae that cause the damage. Proper timing of pest management practices, such as insecticide applications, could be facilitated by the knowledge of the flight activities of *A. ipsilon.*

The pheromone blend of *A. ipsilon* had been reported to be a mixture of (Z)-7-dodecenyl acetate (Z7–12 : Ac) and (Z)-9-tetradecenyl acetate (Z9–14 : Ac) (Hill and Roelofs, 1977; Hill et al., 1979). These components are present at a 5 : 1 ratio in volatile collection from live females (Hill et al., 1979). A 3 : 1 blend of these components is effective in capturing males, performing better than live females (Clement et al., 1981) or blacklight traps (Willson et al., 1981; Levine et al., 1982) in the early flight season of *A. ipsilon.* However, during the late flight season in the northern range, pheromone traps loaded with the 3 : 1 blend are less effective in capturing males than are blacklight traps (Willson et al., 1981; Clement et al., 1982; Levine et al., 1982). The two-component blend is not very effective in France (Causse et al., 1988) or Japan (Wakamura et al., 1986).

Several reports indicate that the addition of (Z)-11-hexadecenyl acetate (Z11–16 : Ac) to the original 2-component blend increases male captures in Japan (Wakamura et al., 1986), France (Causse et al., 1988), and Ukraine (Buleza, 1991). Recently, all three components (Z7–12 : Ac, Z9–14 : Ac, and Z11–16 : Ac), were identified from pheromone gland extracts of female *A. ipsilon* from France, and all three were involved in male attraction as determined from laboratory wind-tunnel tests (Picimbon et al., 1997). Based on these findings, Picimbon et al. (1997) suggested possible pheromone differences between European and North American populations of *A. ipsilon.* Similar differences have been reported in other moth species (e.g., Klun and Cooperators, 1975; Arn et al., 1983; Löfstedt et al., 1986; Peña et al., 1988; Tóth et al., 1992). However, for North

American populations of *A. ipsilon* there is no chemical, behavioral, or trapping data to support or refute a role of Z11–16:Ac in chemical communication.

In the present study we analyzed the pheromone blend of a North American population of *A. ipsilon* to determine if Z11–16:Ac was present. We also investigated the effect of different combinations of Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac on the attraction of male *A. ipsilon* in the field. Finally, we studied the response of laboratory males to the same blends in a wind tunnel.

METHODS AND MATERIALS

Initially, eggs of *A. ipsilon* were obtained from French Agricultural Research Inc. (Lamberton, Minnesota). This colony originated from insects collected in Kansas. Subsequently the colony has been maintained without introduction of feral individuals for over 50 generations. Larvae and ovipositing females were maintained at room temperature under a 14L:10D photoregime. Eggs were placed in groups of 50 in clear plastic boxes 11 cm long × 11 cm wide × 3 cm high half filled with pinto bean diet (Hendrix et al., 1991). The top of the cages had a 4-cm-diameter hole covered with fine mesh nylon screen. After one week, larvae were transferred individually to 2 cm^3 cells half filled with diet and covered with a translucent, breathable construction material (Porex Technology, Fairburn, Georgia). Pupae were separated by sex. Subsequently, males and females were housed in separate environmental chambers under a 16L:8D photoregime at 25°C. Adults were separated daily from the pupae so that their ages were known. They were given access to 10% sucrose–water solution and were considered to be 1 day old during a 24-hr period starting with their first photophase. Voucher specimens have been placed in the University of Kentucky Insect Collection.

Pheromone Gland Extraction and Analysis. The ovipositor and associated pheromone gland of 2- to 4-day-old females were excised during the second half of the scotophase. Noncuticular tissues were removed carefully with fine forceps. Glands were placed in 200 μl of methylene chloride containing 10 ng of internal standard, (*E*,*Z*)-4,7-tridecadienyl acetate (*E*,*Z*-4,7–13:Ac). Gland extracts (23–29 gland equivalents per vial) were kept for 1–2 hr at room temperature and then stored at −80°C. Before analysis, vials were removed from the freezer and allowed to warm to room temperature. Glands were removed, and the volume of the solution was reduced under nitrogen gas to 1–2 μl before injection.

For individual gland extracts, the ovipositors of 2- and 3-day-old females ($N = 24$) were excised and placed individually in 10 μl of methylene chloride containing 1 ng of internal standard. The rest of the procedures were the same as those for pooled samples.

Chemical Analysis. A Hewlett-Packard 5890 Series II Plus gas chromatograph (GC) equipped with either a DB-5 or a DB-Wax column (both columns 30 m × 0.25 mm ID, J & W Scientific, Folsom, California), linked with a Hewlett-Packard 5972 mass selective detector (MSD), was used for analysis of gland extracts. The splitless injector was set at 250°C with the purge valve opening 0.75 min after an injection. GC operating conditions for both columns were the same, with an initial oven temperature of 80°C for 2 min, increasing to 210°C at a rate of 10°C/min. The flow rate of helium through the column was maintained at 1 ml/min. An electronic impact (70 eV) mass spectrum from 40 to 400 amu was obtained. A solution containing 1 ng of Z7-12:Ac, Z9-14:Ac, Z11-16:Ac, and the internal standard was injected to determine the retention times and spectra of the target compounds.

For analysis of individual gland extracts we used a DB-Wax column. We conducted selected ion monitoring focusing on ions characteristic of Z7-12:Ac, *E,Z*-4,7-13:Ac, Z9-14:Ac, and Z11-16:Ac (*m/z*, 166, 178, 194, and 222, respectively). The quantity of each pheromone component was determined by relating its peak area to that of the internal standard and then correcting for the differential responsiveness of the MSD to the compound. This differential responsiveness was established each day by injecting a standard solution containing 1 ng of each compound.

Field Test. Tests were carried out between August 3 and September 10 in 1995, and between June 10 and September 24 in 1996 at the University of Kentucky, Spindletop Research Farm, Lexington. Treatment blends (Table 1) were loaded on rubber septa in 50 μl of solvent at concentrations similar to those used previously by other researchers (Clement et al., 1981; Levine et al., 1982; Wakamura et al., 1986; Causse et al., 1988; Buleza, 1991). The solvent

TABLE 1. PHEROMONE BLENDS EVALUATED IN FIELD AND LABORATORY TESTS

Pheromone blends[a]			Test		
Z7-12:Ac	Z9-14:Ac	Z11-16:Ac	Field 1995	Field 1996	Wind tunnel
✓			✓	✓	✓
✓	✓		✓	✓	✓
✓		✓		✓	✓
	✓	✓			✓
✓	✓	✓	✓	✓	✓
[b]			✓	✓	

[a] Each row is a different pheromone blend. For field tests Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac were applied to rubber septa in amounts of 30, 10, and 60 μg, respectively. For wind-tunnel tests the amounts were 3, 1, and 6 μg, respectively.

[b] Control treatments consisted of 50 μl of hexane loaded onto rubber septa.

was allowed to evaporate for at least 1 hr before the septa were placed in vials to be transported to the field. Rubber septa loaded with 30 μg of Z7–12:Ac, 10 μg of Z9–14:Ac, and 60 μg of Z11–16:Ac emitted these compounds at rates of 16.92 ± 1.05, 0.77 ± 0.06, and 0.62 ± 0.07 ng/30 min, respectively (mean ± SE, $N = 5$) (Gemeno and Haynes, unpublished data, following Baker et al., 1981). Rubber septa were hung from the top of wing sticky traps (Pherocon 1C, Trécé, Salinas, California) 2–4 cm from the sticky bottom. The traps were placed 1.2 m above ground on vertical posts. Distance between traps was 10 m within each block. Four blocks of traps were placed surrounding corn fields. Blocks were separated by at least 50 m. Traps were visited every two to five days in 1995, and every five to nine days in 1996 to count the number of male *A. ipsilon* captured. Rubber septa were replaced 12 and 29 days after the beginning of the test in 1995, and on every visit in 1996. The sticky bottoms were replaced on each visit. The number of males collected per trap per day was transformed [square root of $(n + 1)$] before analysis of variance.

Wind-Tunnel Test. The wind tunnel was modeled after that of Miller and Roelofs (1978). It was a polycarbonate chamber 2 m long × 1 m wide × 1 m high with a fan at one end. The wind tunnel was illuminated from above with three red and three white 25-W incandescent light bulbs. Light intensity, which was kept at the minimum necessary to see the moths, ranged from 12 lux at the top of the tunnel (right underneath the light bulbs), to 1 lux at the bottom of the tunnel. Temperature in the wind tunnel varied from replicate to replicate (22.3°C to 23.9°C), but it varied less than 1°C within each replicate. Wind speed was set at 0.5 m/sec at the center of the tunnel. The pheromone source was placed on a 25-cm-high metal platform (source platform) that was located 30 cm from the upwind end of the tunnel. Males were released individually from the top of a 25-cm-high metal platform (release platform) 1.5 m downwind of the source platform. Smoke from a cigarette showed that the pheromone plume was filamentous and broadened from a diameter of ca. 1 cm at the source platform to ca. 10 cm at the point of male release.

Treatment blends (Table 1) were loaded on rubber septa, and the solvent was allowed to evaporate for at least 1 hr before the test began. Rubber septa were placed on the top of a 1-ml plastic pipet placed vertically on the source platform. Each treatment blend was presented consecutively to four individual males before a new treatment was used. Each treatment septum was used for only one night. Between 10 and 15 hr before the scotophase of observation, males were placed individually in wire cages 4 cm wide × 8 cm high provided with 10% sugar water on a cotton pad. Males were kept in the darkened wind tunnel room between 5 and 50 min before the test; otherwise, they remained in the dark in an environmental chamber. Male response was observed during the second half of the scotophase, which corresponds with the period of female calling (Swier et al., 1977).

After release, male behavior was observed for 2 min. Behaviors recorded included staying on the platform, taking flight, locking-on (zigzagging or hovering in the pheromone plume), oriented flight (upwind zigzagging flight on the pheromone plume), distance of closest approach to the pheromone source, and contacting the pheromone source. Ryan's multiple range test for proportions (Ryan, 1960) was used to compare the different responses to treatments (N = 36–44 per treatment).

RESULTS

Pheromone Identification. In the gland extracts, peaks with retention times identical to Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac were observed from both DB-5 and DB-Wax columns. Mass spectra of the three peaks from the pheromone gland extracts were identical to mass spectra of Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac from the standard solution. The Z7–12:Ac peak from the standard solution and the peak corresponding in retention time with Z7–12:Ac in the sample were characterized by prominent ions at *m/z* 55, 67, 81, 96, and 109, as well as less abundant ions at *m/z* 61 (indicating the acetate group) and 166 (characteristic of 12-carbon monounsaturated acetates). The peak of Z9–14:Ac from the standard solution and the peak corresponding in retention time with Z9–14:Ac in the sample were characterized by prominent ions at *m/z* 55, 67, 82, 96, and 110, as well as less abundant ions at *m/z* 61 and 194 (a characteristic ion for 14-carbon monounsaturated acetates). Z11–16:Ac and the peak corresponding in retention time with Z11–16:Ac from the sample were characterized by prominent ions at *m/z* 55, 67, 82, 96, and 110, and less abundant ions at *m/z* 61 and 222 (a characteristic ion for 16-carbon monounsaturated acetates).

Quantities of Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac detected in individual females were (mean ± SE) 0.144 ± 0.013, 0.030 ± 0.004, and 0.036 ± 0.008 ng, respectively. The percentages of Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac in pheromone gland extracts from individual females were (mean ± SE) 70.5 ± 2.58:14.24 ± 0.82:15.26 ± 2.19, respectively.

Field Test. Only pheromone traps baited with Z7–12:Ac and Z9–14:Ac (two-component blend) or with Z7–12:Ac, Z9–14:Ac, and Z11–16:Ac (three-component blend) were effective in capturing males (Figures 1 and 2). The other pheromone blend treatments and the control traps did not capture any male *A. ipsilon*. Significantly more males were captured in traps with the three-component blend than the two-component blend both in 1995 and 1996 (df = 1,86, F = 10.44, P = 0.001 and df = 1,118, F = 30.45, $P < 0.001$, respectively; ANOVA; Figures 1 and 2). The superior trapping effectiveness of the three-component blend was observed throughout the sampling period in both years. During the first half of the sampling period in 1995, captures were at their

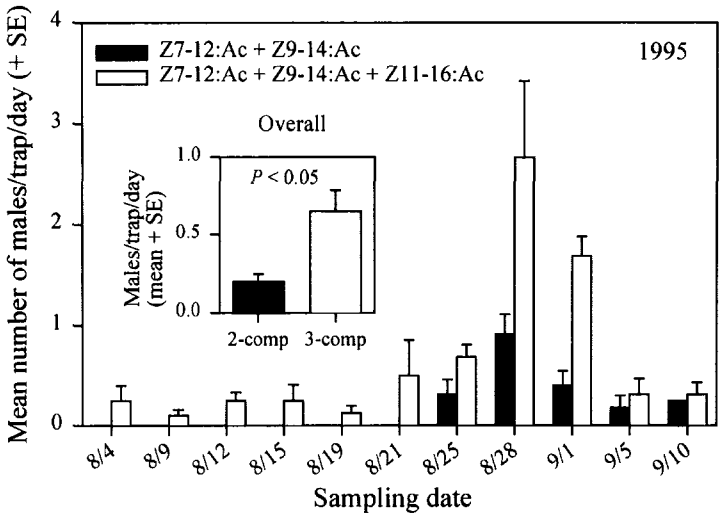

FIG. 1. Field captures of male *Agrotis ipsilon* in pheromone traps baited with a two-component blend (Z7-12:Ac and Z9-14:Ac) or a three-component blend (Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac). Control (hexane) and Z7-12:Ac traps captured no males. Inset shows means (+SE) for the entire period from August 3 to September 10, 1995.

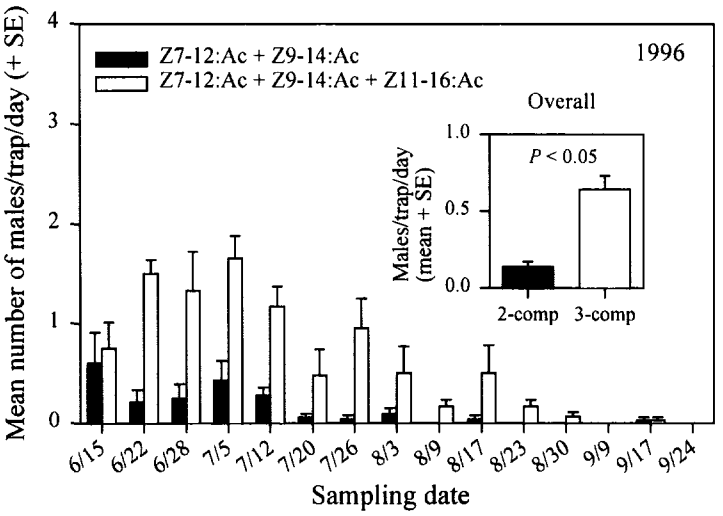

FIG. 2. Field captures of male *Agrotis ipsilon* in pheromone traps baited with a two-component blend (Z7-12:Ac and Z9-14:Ac) or a three-component blend (Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac). Control (hexane), Z7-12:Ac, and Z7-12:Ac plus Z11-16:Ac traps captured no males. Inset shows means (+SE) for the entire period from June 10 to September 24, 1996.

lowest, and males were trapped only with the three-component blend (Figure 1). During the second half of the sampling period in 1996, trap captures declined, and on some days males were collected only in traps baited with the three-component blend (Figure 2).

A peak in capture rates of *A. ipsilon* was observed in 1995 between August 21 and September 1. During this same period in 1996, the male capture rate was low. In 1996, when traps were run for a longer seasonal period, a broad peak in the male-capture rate was observed that was at its greatest between June 28 and July 5.

On the last sampling date in 1996 males of the dingy cutworm, *Feltia jaculifera* (Guenée), were captured in traps baited with the three-component blend. Each of the four three-component blend traps caught individuals of this species (four, four, three, and two individuals). Voucher specimens of this species have been placed in the University of Kentucky Insect Collection.

Wind Tunnel Test. Significantly more males initiated a response [locking-on (LO)] to the three-component blend (*Z*7–12 : Ac, *Z*9–14 : Ac, and *Z*11–16 : Ac) or to a two-component blend containing *Z*7–12 : Ac and *Z*9–14 : Ac than to any other blends tested (Figure 3A, $P < 0.05$; Ryan's multiple comparison test for proportions). There was no significant difference in the proportion of males that began to orient upwind (OF) to this two-component blend or to the three-component blend ($P > 0.05$). However, significantly fewer males oriented closer than 10 cm to the source (OF < 10 cm) for this two-component blend than the full three-component blend ($P < 0.05$). The overall rate of source contact (SC) was 2.27 times greater for the three-component blend than for a blend of *Z*7–12 : Ac and *Z*9–14 : Ac. No other blends stimulated source contact.

A conditional probability analysis indicated that failure to contact the pheromone source in response to *Z*7–12 : Ac; *Z*7–12 : Ac and *Z*11–16 : Ac; and *Z*9–14 : Ac and *Z*11–16 : Ac occurred early in the behavioral sequence (Figure 3B). The improved response to the three-component blend compared to the best two-component blend (*Z*7–12 : Ac and *Z*9–14 : Ac) could not be attributed to any individual transition, but the results from the three-component indicated a trend for improved responsiveness at every behavioral transition [taking flight (TF) >> LO, LO >>OF, OF >> OF < 10 cm, and OF < 10 cm >> SC].

DISCUSSION

Chemical analysis of pheromone gland extracts indicates that females from a North American population of *A. ipsilon* produce *Z*11–16 : Ac in addition to *Z*7–12 : Ac and *Z*9–14 : Ac. The importance of *Z*11–16 : Ac as a pheromone blend component was demonstrated both in the field and in a wind tunnel. Together these chemical and behavioral data support the identification of *Z*11–16 : Ac as a third pheromone component in a North American population of *A. ipsilon.*

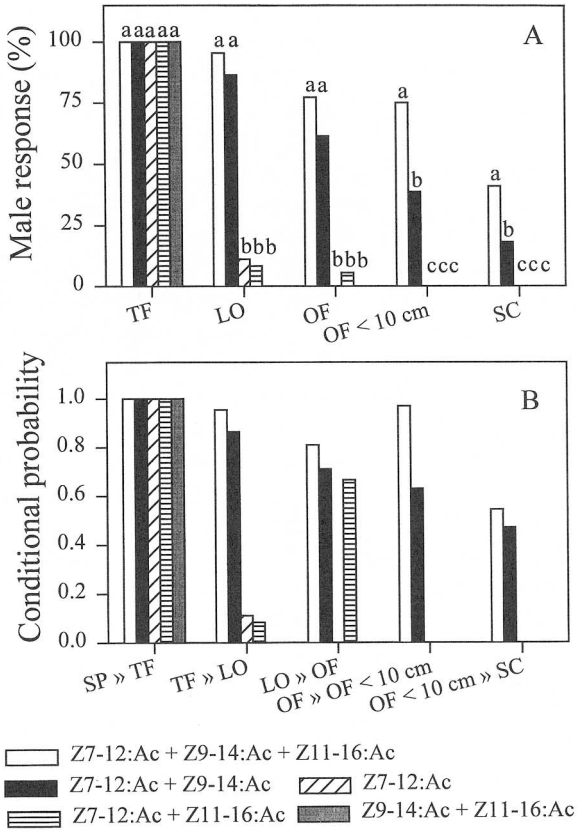

FIG. 3. Wind-tunnel responses of male *Agrotis ipsilon* to pheromone blends containing various combinations of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac. Behaviors observed were: staying on the platform (SP), taking flight (TF), locking-on to the pheromone plume (LO), orient upwind (OF), making it to within 10 cm of the source (OF < 10), and contacting the source (SC). (A) Percentage of males exhibiting the observed behaviors. Treatments within each behavioral category that are significantly different do not share the same letters ($P < 0.05$; Ryan's multiple comparison test for proportions). (B) Conditional probability of making the indicated behavioral transition (e.g., LO >> OF indicates probability of making the transition between locking on to the pheromone plume and oriented flight).

The addition of Z11-16:Ac to the previously identified two-component blend of Z7-12:Ac and Z9-14:Ac improved trap capture rate in the field. This improvement was probably due to the fact that males were more likely to orient toward and approach the source of the three-component blend, as is seen in the wind tunnel test. In the wind tunnel, the three-component blend elicited an

improved response at each behavioral transition, suggesting that the blends act as a unit for all stages of the behavior. In the field, it is possible that the three-component blend may elicit responses at greater distances from the pheromone source, as is the case for the full pheromone blend of the Oriental fruit moth, *Grapholita molesta* (Busck) (Linn et al., 1987).

Similar increased effectiveness of the three-component blend compared with the two-component blend has been observed in Japan (Wakamura et al., 1986), France (Causse et al., 1988), and Ukraine (Buleza, 1991). French *A. ipsilon* moths produce Z11-16:Ac in addition to Z7-12:Ac, Z9-14:Ac, (Z)-11-tetradecenyl acetate (Z11-14:Ac) and (Z)-11-hexadecenol (Z11-16:OH) (Picimbon et al., 1997). However, of all these compounds, only Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac are important in eliciting male attraction in the wind tunnel (Picimbon et al., 1997). Additional components may be involved in the chemical communication system of North American *A. ipsilon.*

Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac are present together in the pheromone blend of 14 moth species distributed among four subfamilies in the family Noctuidae (Arn et al., 1997). In our study, male *F. jaculifera* were attracted to traps baited with the three-component blend. In North America, *F. jaculifera* probably consists of two sibling species that are morphologically indistinguishable but produce two different blend ratios of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac (Byers and Struble, 1990). Electroantennograms from field-collected males demonstrated the existence of at least two male phenotypes, each responding preferentially to one of the two pheromone types (Byers et al., 1990). Male *F. jaculifera* were not captured in the same trapping period as *A. ipsilon* in 1996. In fact, only two male *A. ipsilon* (0.66% of the entire catch) were captured within 20 days of the date on which all of the male *F. jaculifera* were observed. Seasonal differences between *A. ipsilon* and *F. jaculifera* may contribute to reproductive isolation between these two species. However, additional unidentified pheromone components could also contribute to the species specificity of the signal.

In the northern range of occurrence of *A. ipsilon*, there is seasonal variation in the relative efficiency of pheromone traps and blacklight traps. Early in the season, pheromone traps are more efficient than blacklight traps. Late in the season, pheromone traps attract very few males, whereas blacklight traps attract many *A. ipsilon*, including both males and females (Willson et al., 1981; Clement et al., 1982; Levine et al., 1982). Seasonal delayed reproduction, related to migration, has been suggested as an explanation for this observation (Kaster and Showers, 1982; Clement et al., 1985). Because we have found that the three-component blend is far more effective than the two-component blend that was used in these earlier studies, it would be worthwhile to reexamine the seasonal changes in male responsiveness to pheromone traps, and their relationship to

delayed reproduction and long-range movement with the full, newly identified blend.

The three-component pheromone blend should be more effective in detecting the presence of a population of *A. ipsilon* than the two-component blend that is currently available commercially. In a migrant species such as *A. ipsilon*, the magnitude and date of the initial influx of moths into more northern areas establishes in part the potential for the development of a serious pest population. If this influx is early and/or involves numerous individuals, then outbreaks later in the season could be more likely. The three-component blend should be more likely to detect the presence of new immigrants. In addition, throughout the season the three-component blend, by being a more effective sampler of male population levels, should be a better predictor of subsequent larval population levels. Thus, the new pheromone blend could be useful in both detecting and monitoring populations of *A. ipsilon*, but more research is required to establish the relationships between sampled adult male levels and subsequent larval population levels.

In addition to the potential practical value of the three-component pheromone blend, improved pheromone traps could be useful in characterizing the nature of migration in *A. ipsilon*. This is one of very few species in which both northern and southern migration have been demonstrated (Showers, 1997). The evidence for this complete migration is strong, but is based on recapture of only seven individuals in pheromone traps. A more effective pheromone blend could help to characterize temporal and geographical patterns of migration and the related seasonal changes in population densities.

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